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Mobility of pigment-protein complexes in Cyanobacteria

A thesis submitted for the degree of Doctor of Philosophy

Sarah Joshua

Department of Biology

University College London

University of London

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

I dedicate this to those who believed I could. They know who they are.

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To my sisters who have tolerated my moods, and most specifically to my mother, I shall be eternally grateful. Without their encouragement, this body of research would never have been produced.

Abstract

Phycobilisomes, the light harvesting complexes of cyanobacteria are highly mobile, fluorescent complexes known to diffuse freely on the thylakoid membranes, interacting with the reaction centre complexes to mediate efficient photosynthesis. The primary aim of this project is to establish what processes require this rapid movement of the complexes using a number of genetic, biochemical and microscopic techniques.

The cyanobacterial species used extensively in the work presented in this thesis are the fully sequenced, naturally transformable *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7942. The latter lends itself particularly well to quantitatively elucidating the diffusion rate of fluorescent complexes, but qualitative detection of mobile fluorescent complex is also feasible with *Synechocystis* 6803.

State transitions are observed in cyanobacteria upon the alteration of illumination conditions. A rapid redistribution of excitation energy between the reaction centres is observed. This was investigated using high osmotic strength buffers to fix phycobilisomes to reaction centres they were associating with upon their addition, thus inhibiting their mobility, as adjudged by spectroscopy and microscopy using the Fluorescence Recovery after Photobleaching (FRAP) technique. It was found that mobile phycobilisomes are required for cells to be capable of state transitions.

Non-Photochemical Quenching (NPQ) is a protective mechanism seen in iron-deprived cyanobacteria. Extensively studied in plants, its supposed function is to dissipate excess energy as heat to prevent photodamage to the reaction centres. Using *Synechocystis* 6803 and the techniques described above, phycobilisome mobility was determined to be critical to NPQ induction, and the interaction with IsiA in cyanobacteria was proposed as being involved in the process.

A previously inactivated gene thought to be involved in state transitions, *rpa*C, was over-expressed in *Synechocystis* 6803 and knocked out in *Synechococcus* 7942 and gave pleiotropic effects. The conclusion that the binding of phycobilisomes to PSII

is predictably stronger than to PSI was exploited by comparing the strength of the binding in the Synechococcus 7942 mutant with the wild type. Data were suggestive of the protein being involved in phycobilisome to PSII binding.

Psb28$^-$ mutants of both species used in this thesis were extensively characterised, as the cells also presented a highly unusual mobile PSII phenotype. Psb28 is possibly involved in maintaining thylakoid membrane organisation.

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List of Abbreviations

A_0	Primary electron acceptor chlorophyll of PSI
A_1	Phyloquinone
AFM	Atomic Force Microscope
APC	Allophycocyanin
ATP	Adenosine triphosphate
AU	Arbitrary units
CCA	Complementary Chromatic Adaptation
Chl	Chlorophyll
Cm^R	Chloramphenicol resistance cassette
Cytbf	Cytochrome bf complex
DBMIB	2,5-dibromo-3-methyl-6-isopropyl-1,4-benzoquinone
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide 5'-triphosphate
EDTA	Ethylene diamine tetra-acetic acid
EM	Electron microscopy
EPR	Electron paramagnetic resonance
ETC	Electron Transport Chain
F_0	Initial (baseline) fluorescence of dark adapted cells
F_A	Iron-sulphur centre F_A
F_B	Iron-sulphur centre F_B
F_v	Variable fluorescence
F_X	Iron-sulphur centre F_X
FRAP	Fluorescence Recovery After Photobleaching
g	Gravitational acceleration force
GFP	Green Fluorescent Protein
GT	Glucose tolerant
HEPES	N-(2-hydroxyethyl)piperazine-N'-(ethanesulfonic acid)
Hr	Hour
IPTG	IsopropylBeta-D-thiogalactopyranoside
Kan^R	Kanamycin resistance cassette
kb	Kilobases
LB	Luria-Bertani medium
LHCI	Light-harvesting complex I
LHCII	Light-harvesting complex II
µE	MicroEinsteins
µg	Microgram
µl	Microlitre
µm	Micrometre
µM	Micromolar
µmol	Micromoles
µs	Microsecond
mg	Milligram
min	Minute
ml	Millilitre

mM	Millimolar
MOPS	3-(N-morpholino)propanesulfonic acid
NADP	Nicotinamide adenine dinucleotide phosphate
nM	Nanomolar
NPQf	Non-photochemical quenching of fluorescence
ns	Nanosecond
P680	Primary electron donor of PSII
P700	Primary electron donor of PSI
PBQ	Phenyl-1,4-benzoquinone
PBS	Phycobilisome
PC	Phycocyanin
PCC	Pasteur Culture Collection
PCR	Polymerase chain reaction
PE	Phycoerythrin
PEC	Phycoerythrocyanin
pH	$-\log_{10}$ of the hydrogen ion concentration
PQ	Plastoquinone
PRK	Phosphoribulokinase
PSI	Photosystem I
PSII	Photosystem II
psi	Pounds per square inch
PAM	Pulse Amplitude Modulated
Q_o	Quinone binding site within cytochrome bf complex
***rpa*C**	Regulator of phycobilisome association, C gene
rpm	Revolutions per minute
RNA	Ribonucleic acid
RubisCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
s	Second
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SE	Standard error
$Spec^R$	Spectinomycin resistance cassette
SPCM	0.5 M $KHPO_4/KH_2PO_4$, 0.5 M sucrose, 0.3 M sodium citrate, pH 6.8
***S.* 6803**	Synechocystis sp. PCC 6803
***S.* 7942**	Synechococcus sp. PCC 7942
Tris	Tris(hydroxymethyl)aminomethane
TES	N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid
w/v	Weight per unit volume
WT	Wild type
X-gal	5-bromo-4-chloro-indolyl-β -D-galactosidase

mM	Millimolar
MOPS	3-(N-morpholino)propanesulfonic acid
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
nM	Nanomolar
NPQf	Non-photochemical quenching of fluorescence
ns	Nanosecond
P680	Primary electron donor of PSII
P700	Primary electron donor of PSI
PBQ	Phenyl-1,4-benzoquinone
PBS	Phycobilisome
PC	Phycocyanin
PCC	Pasteur Culture Collection
PCR	Polymerase chain reaction
PE	Phycoerythrin
PEC	Phycoerythrocyanin
pH	$-\log_{10}$ of the hydrogen ion concentration
PQ	Plastoquinone
PRK	Phosphoribulokinase
PSI	Photosystem I
PSII	Photosystem II
psi	Pounds per square inch
PAM	Pulse Amplitude Modulated
Q_o	Quinone binding site within cytochrome bf complex
***rpa*C**	Regulator of phycobilisome association, C gene
rpm	Revolutions per minute
RNA	Ribonucleic acid
RubisCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
s	Second
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SE	Standard error
$Spec^R$	Spectinomycin resistance cassette
SPCM	0.5 M $KHPO_4/KH_2PO_4$, 0.5 M sucrose, 0.3 M sodium citrate, pH 6.8
***S.* 6803**	Synechocystis sp. PCC 6803
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Tris	Tris(hydroxymethyl)aminomethane
TES	N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid
w/v	Weight per unit volume
WT	Wild type
X-gal	5-bromo-4-chloro-indolyl-β -D-galactosidase

Chapter 1: Introduction

Chapter 1: Introduction

1.1. Cyanobacteria

Cyanobacteria are oxygenic photoautotrophs and belong to one of eleven eubacterial phyla. It is widely believed that cyanobacteria formed the precursors of green plant chloroplasts by virtue of a symbiotic event (see Palmer, 2003 for review), whereby a photosynthetic eukaryote evolved.

Microfossils presumed to be cyanobacterium-like have been dated as approximately 3.5 billion years old (Schopf and Packer, 1987). The rise of oxygen levels in Earth's primitive atmosphere around 2.7 billion years ago coincides with the wide-spread establishment of cyanobacteria (Des Marais, 2000). They would become a dominant phylum owing to the toxicity of oxygen gas (generated by photolysis of water in the process of photosynthesis) to certain other eubacterial phyla (Nitschke *et al*, 1998), eventually being incorporated into non-photoautotrophic cells by symbiosis some 0.6 billion years later. The symbiosis has resulted in eukaryotic photoautotrophs, where the plastid is under the autonomy of the eukaryote's nucleus, and mechanisms alternative to that of cyanobacterial photosynthesis have evolved over the past 2 billion years. A possible sequence of events is summarised in figure 1.1.

The earliest oxygenic photosynthetic species, the cyanobacteria (see figure 1.2, for example), possess phycobilisomes for light harvesting to the two photosynthetic reaction centres I and II. This is, however, not universal, as *Prochlorococcus*, a marine cyanobacterium uses a complex divinyl chlorophyll *b* light harvesting antenna. The diversity is maintained since *Prochlorococcus* appears to have a selective advantage over its phycobilisome-utilising counterparts under conditions of iron stress (Ting *et al*, 2002).

The study of the cyanobacterial photosynthesis is aided by the fact that some species are naturally transformable, taking up exogenous DNA with relative ease,

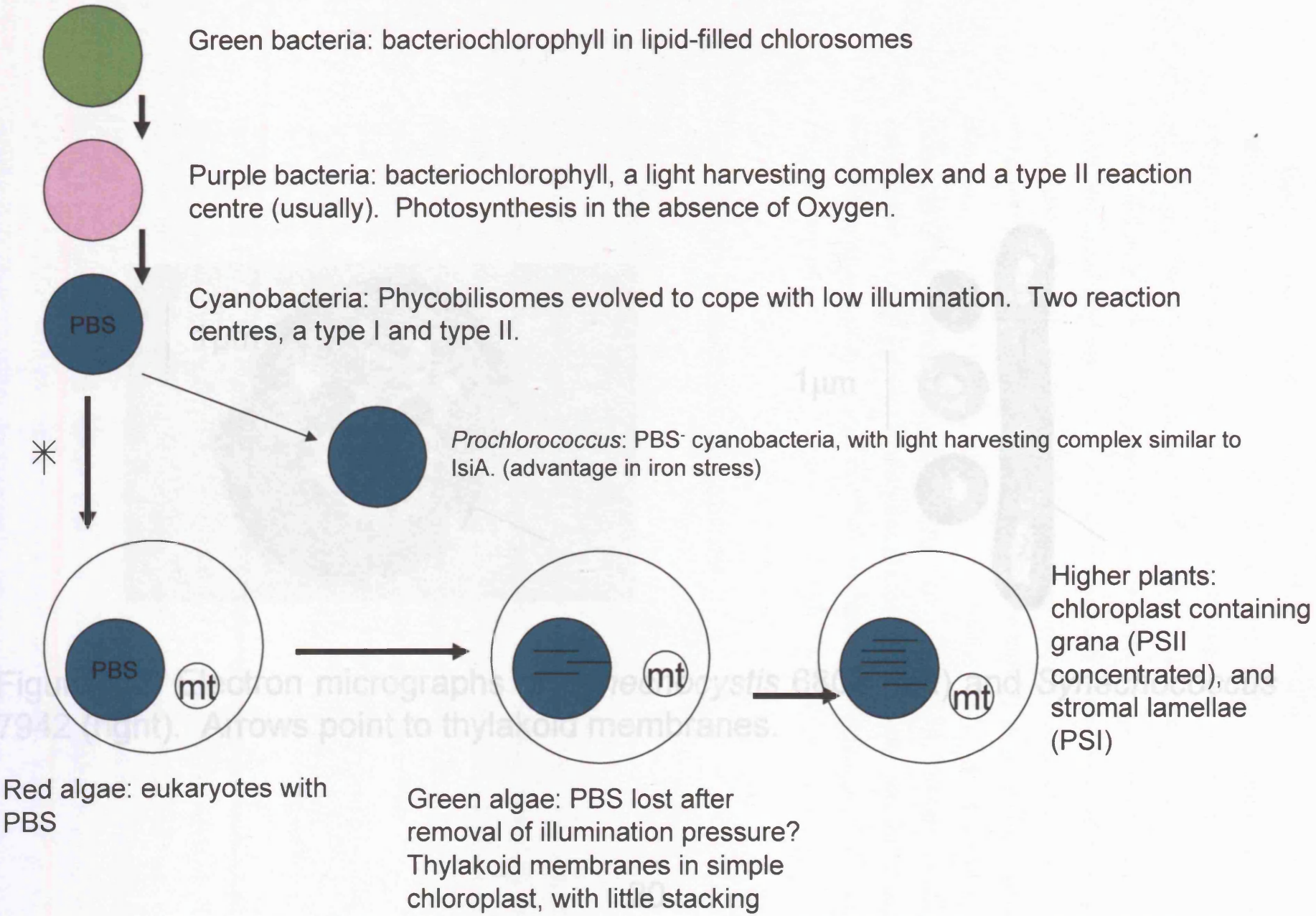

Figure 1.1. Stepwise speciation of phototrophic organisms. Asterisk indicates symbiotic event, mt indicates mitochondria present, PBS=phycobilisomes. Theory from Mullineaux, 2005

Figure 1.2. Electron micrographs of *Synechocystis* 6803 (left) and *Synechococcus* 7942 (right). Arrows point to thylakoid membranes.

Synechocystis 6803 and *Synechococcus* 7942 for example. Others, *Thermosynechococcus elongatus* included, are transformable by electroporation, (Mullenhoff and Chauvat, 1996), though recently it has been shown that this species may also undergo natural transformation (Onai *et al*, 2003). Subsequently, plasmid vectors can undergo homologous recombination with the bacterial genome. This is useful in generating inactivation and over-expression mutants, in addition to fluorescently tagged ones, particularly since *Synechocystis* 6803, *Synechococcus* 7942 and *Thermosynechococcus elongatus* have completely sequenced genomes (Kaneko and Tabata, 1997, http://genome.jgi-psf.org/draft_microbes/synel/synel.home.html and Nakamura *et al*, 2002, respectively). For the purposes of analysing photosynthetic membranes, the simply arranged thylakoids of *Synechococcus* 7942 are particularly useful (Sarcina *et al*, 2001, for example).

1.2. Overview of Cyanobacterial Photosynthesis

1.2.1. The Light-Dependent Reaction

This involves the photolysis of water, ultimately to generate ATP and reduced $NADP^+$ via a complex cascade of redox reactions. Light is required for this to occur, and is harvested in cyanobacteria mainly by phycobilisomes (described in section 1.3.), and channelled towards the photosynthetic reaction centres, photosystem I and II. Chlorophyll is also important for harvesting to PSI.

1.2.1a. Photosystem I

Photosystem I (PSI) is a membrane bound iron-sulphur-type reaction centre whose function is photo-conversion (see Golbeck, 1994 and Chitnis, 2001, for review). It consists of a multiple-protein complex and can be classified as a plastocyanin:ferredoxin oxidoreductase. Photo-conversion requires three key processes to occur: first, the capture of photons via a photochemical trap, in this case P700, a chlorophyll-containing molecule encoded by psaA and PsaB genes. Peripheral polypeptides within the reaction centre complex function to stabilise the

charge separation that is to occur and to facilitate the conversion of $NADP^+$ to NADPH. Charge separation takes place by virtue of a cascade of redox reactions, culminating in the reduction of stroma-soluble ferredoxin and the corresponding oxidation of plastocyanin. The quantum efficiency of this process is high, but only an estimated 43% of the captured energy results in photochemical conversion.

P700 accepts the light energy harvested by the phycobilisomes and donates an electron to A_0, a chlorophyll monomer (Mathis and Setif, 1988; Mathis, *et al*, 1988; Kim *et al*, 1989), which in turn reduces A_1, now established to be a phylloquinone molecule (Petersen *et al*, 1987). The electron continues down a redox pathway in the direction of the stroma, first via a [4Fe-4S] cluster, later to F_A and F_B, both iron-sulphur clusters themselves, before ferredoxin and flavodoxin at the stromal interface can be reduced. Electron transport through PSI is depicted in figure 1.3.

The structure of PSI has been elucidated to 4.4 angstrom resolution in higher plants (Ben-Shem *et al*, 2003) and to 2.5 angstrom resolution in the cyanobacterium Thermosynechococcus elongatus. That the 3-dimensional structures of many of the integral electron donors and acceptors have been resolved (plastocyanin, ferredoxin, flavodoxin and the oxidoreductase included) in addition to many of the polypeptide subunits of the reaction centre itself have resulted in a more comprehensive understanding of cyclic and non-cyclic electron transport.

1.2.1b. Photosystem II

Photosystem II (PSII) is a membrane-bound dimeric complex and is classified as a quinone-type reaction centre, acting to oxidise water by the process of photo-conversion and ultimately bring about the reduction of $NADP^+$ and plastoquinone (reviewed by Blankenship, 2002). P680, excited by light energy donates an electron to pheophytin, which will consequently be capable of reducing Q_A, a plastoquinone. Within PSII, quinone molecules act as a two-electron gate. A dual electron acceptor, also a quinone, and termed Q_B, is reduced by Q_A^-. The cation produced by the reduction of pheophytin is re-reduced by Z, a tyrosine residue. The centre for the photolysis of water, the 4Mn cluster, becomes oxidised in order to permit this reduction process, the electrons being derived from the reaction

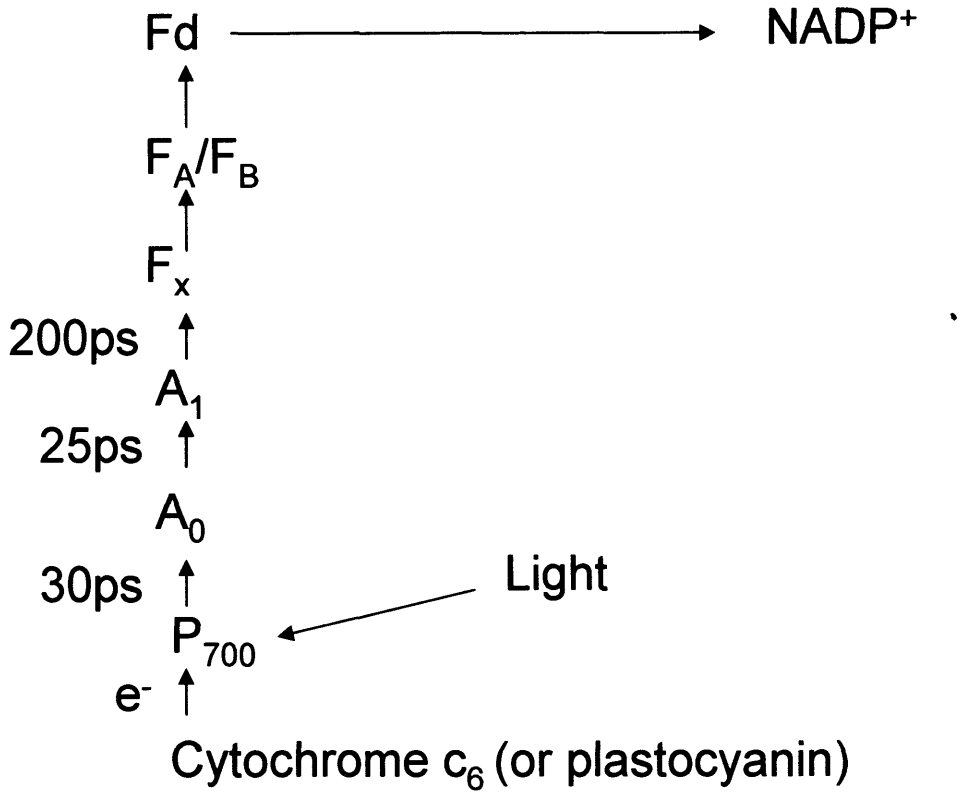

Figure 1.3. Electron flow through Photosystem I. Times given in picoseconds (ps) denote approximate time taken for electron transfer. Refer to list of abbreviations for details of electron carriers.

$$2H_2O \rightarrow 4H^+ + O_2 + 4e^-.$$

A lot of progress has been made recently in elucidating the structure of the 650kDa PSII reaction centre in cyanobacteria to high resolution. Most recently, it was obtained to 3.5 angstrom resolution (Ferreira *et al*, 2004). Photosystem II also possesses carotenoids which act to prevent damage to the reaction centre by quenching triplet excited state chlorophylls before they react with oxygen and cause damage. Photo-conversion in PSII is summarised in figure 1.4.

1.2.2. The Light-Independent Reaction

Both cyanobacteria and green plants depend upon carbon dioxide gas as their primary carbon source for synthesising organic metabolically important compounds, including carbohydrates, lipids and proteins. The light-independent reactions of photosynthesis utilise the products of the light-dependent processes, NADPH (or another strong reducing agent) and ATP, in order to fix carbon and hence synthesise these complex compounds, against a steep thermodynamic gradient.

Carbon dioxide must first be reduced via the Calvin cycle, a reductive pentose phosphate pathway. Two enzymes, unique to the Calvin process, and critical in function to this biosynthetic procedure are phosphoribulokinase (PRK) and ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO), the latter having been reported to be the most abundant protein on Earth (Ellis, 1979). The first phosphorylates the 5-carbon sugar, ribulose-5-phosphate, whereupon the carboxylase activity of RubisCO then acts to fix the carbon dioxide molecule to the resultant ribulose-1,5-bisphosphate (RuBP). This results in the production of two molecules of the 3-carbon organic intermediate, 3-phosphoglyceric acid.

RubisCO is synthesised by photosynthetic organisms in large quantities, and this observation is attributed to its poor catalytic turnover, fixing between 1000 and 2000 moles of carbon dioxide per mole of enzyme per minute, depending upon the species under investigation (Tabita, 1994). In cyanobacteria, the type I RubisCO is composed of eight large and eight small subunit polypeptides, encoded in an operon

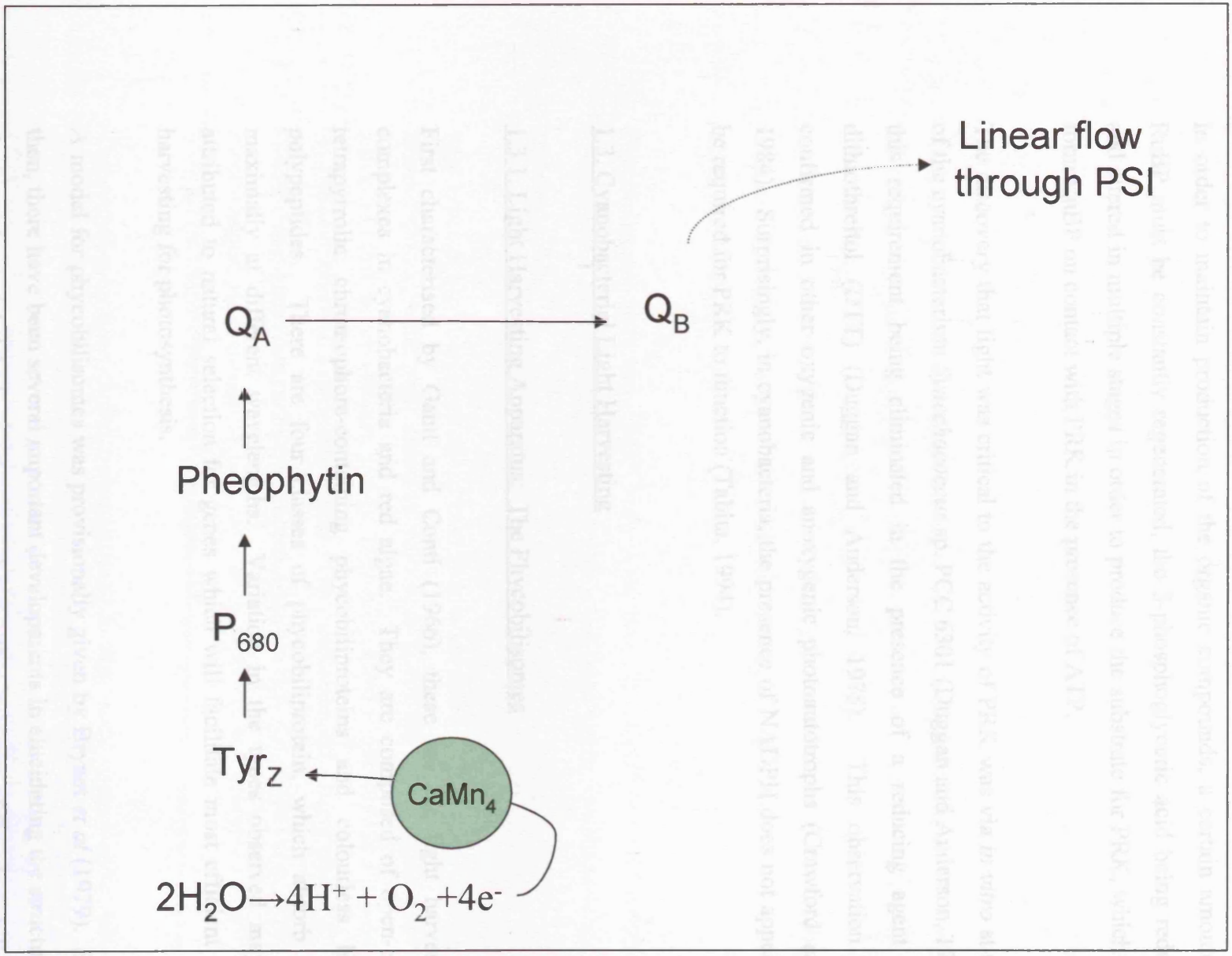

Figure 1.4. Photoconversion and electron flow through Photosystem II. Refer to list of abbreviations for details of electron carriers

by *rbc*L and *rbc*S genes respectively (Tabita, 1987), a transcriptional unit that also possesses a gene for the activation of RubisCO, dubbed *rca*.

In order to maintain production of the organic compounds, a certain amount of RuBP must be constantly regenerated, the 3-phosphoglyceric acid being reduced and altered in multiple stages in order to produce the substrate for PRK, which will form RuBP on contact with PRK in the presence of ATP.

The discovery that light was critical to the activity of PRK was via *in vitro* studies of the cyanobacterium *Synechococcus* sp. PCC 6301 (Duggan and Anderson, 1975), this requirement being eliminated in the presence of a reducing agent like dithiothreitol (DTT) (Duggan and Anderson, 1975). This observation was confirmed in other oxygenic and anoxygenic photoautotrophs (Crawford *et al*, 1984). Surprisingly, in cyanobacteria, the presence of NADPH does not appear to be required for PRK to function (Tabita, 1994).

1.3. Cyanobacterial Light Harvesting

1.3.1. Light Harvesting Apparatus: The Phycobilisomes

First characterised by Gantt and Conti (1966), these are the light harvesting complexes in cyanobacteria and red algae. They are composed of open-chain tetrapyrrolic chromophore-containing phycobiliproteins and colourless linker polypeptides. There are four classes of phycobiliprotein, which absorb light maximally at different wavelengths. Variation in the types observed may be attributed to natural selection for genes which will facilitate most efficient light harvesting for photosynthesis.

A model for phycobilisomes was provisionally given by Bryant *et al* (1979). Since then, there have been several important developments in elucidating the structure of the cyanobacterial light harvesting complexes. The structure of the phycobilisome can be variable across species, but they primarily consist of a two or three-cylinder allophycocyanin (APC, A_{max} at λ=650nm) core with six phycocyanin (PC, A_{max} at

λ=615-640nm) rods radiating from it (see figure 1.5). Some species contain phycoerythrin (PE, A_{max} at λ=565-575nm) and/or phycoerythrocyanin (PEC, A_{max} at λ=575nm), both of which, if present, will be located on the rods distal to the APC core. *Synechocystis* 6803 does not contain PE or PEC in its phycobilisomes, but these phycobiliproteins are present in certain *Synechococcus* species, and may in part explain why certain marine species of *Synechococcus* have been observed to be able to maintain optimal rates of photosynthesis at extremely low light intensities (Kursar *et al*, 1981).

The phycobilisome is assembled anchored to the cytoplasmic side of the thylakoid membranes by linker polypeptides (Capuano *et al*, 1991; 1993). There are four classes of linker: the L_R assembles the phycobiliproteins to make the rods and the L_{RC} attaches those rods to the core, which itself is assembled by the L_C. The L_{CM} mediates interaction between the core and the thylakoid membrane (see Sidler, 1994, for review).

Phycobilisome structure has been resolved by electron microscopy for *Synechocystis* 6803 (Elmorjani *et al*, 1986), but once again, the shape is variable across species.

Four types of phycobilisome shape have been observed:
Hemidiscoidal is the most common with several species including *Synechocystis* exhibiting this morph (Glazer, 1984). Hemiellipsoidal is observed in *Porphyridium cruentum* (Gantt and Lipschultz, 1972). The bundle-shaped variety observed in the Gleobacter which have no thylakoid membranes is rare (Guglielmi *et al*, 1981) and block-shaped phycobilisomes have also been reported, for example in *Griffithsia pacifica* (red alga) (Gantt and Lipschultz, 1980).

The cyanobacteria which feature prominently in this thesis, *Synechocystis* 6803 and *Synechococcus* 7942 have phycobilisomes which differ only in the number of core cylinders, *Synechococcus* 7942 having one less than the three of *Synechocystis* 6803 (Glazer, 1984, for review).

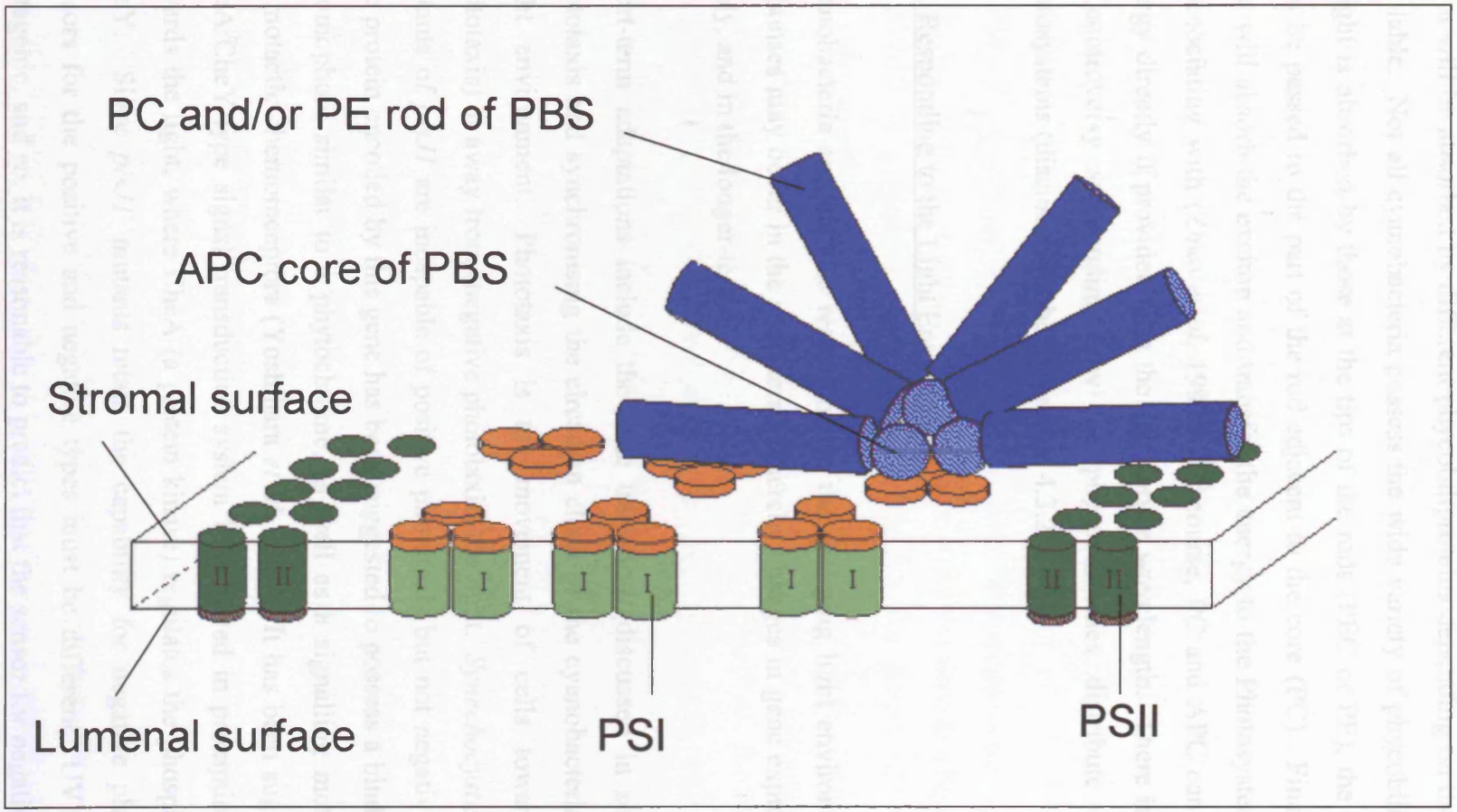

Figure 1.5. Cartoon of hemidiscoidal phycobilisome on surface of thylakoid membrane.
Rows of PSII dimers in the thylakoid membrane with trimeric PSI are shown. The phycobilisome is able to interact with both via the allophycocyanin (APC) core. Light can also be harvested by phycocyanin (PC) and/or phycoerythrin (PE), located in the rods. Courtesy of C. Mullineaux, Queen Mary College, London

1.3.2. The Mechanism of Cyanobacterial Light Harvesting

Light will be absorbed by different phycobiliproteins depending on the wavelength available. Not all cyanobacteria possess the wide variety of phycobiliproteins, but if light is absorbed by those at the tips of the rods (PEC or PE), the excitons will then be passed to the part of the rod adjacent to the core (PC). Finally, the APC core will absorb the exciton and transfer the energy to the Photosystem complex it is associating with (Zhao *et al*, 1992). Of course, PC and APC can absorb light energy directly if provided with the appropriate wavelength. There is a great deal of controversy surrounding how the phycobilisomes distribute light to the photosystems (discussed further in section 4.3.2.).

1.4. Responding to the Light Environment

Cyanobacteria are adept at responding to the changing light environment. These responses may occur in the short-term, whereby changes in gene expression do not apply, and in the longer-term.

Short-term adaptations include the state transition (discussed in section 1.4.3), phototaxis and synchronising the circadian clock of the cyanobacteria to the day-night environment. Phototaxis is the movement of cells towards (positive phototaxis) or away from (negative phototaxis) the light. *Synechocystis* inactivation mutants of *pisJ1* are incapable of positive phototaxis but not negative phototaxis. The protein encoded by this gene has been suggested to possess a binding site for a chromophore similar to a phytochrome, as well as a signalling motif similar to chemotactic chemoreceptors (Yoshihara *et al*, 2000). It has been suggested that a CheA/CheY-type signal transduction system is involved in prompting movement towards the light, where CheA (a protein kinase) regulates the phosphorylation of CheY. Since *pisJ1*$^-$ mutants retain the capability for negative phototaxis, the sensors for the positive and negative types must be different. UV light can be mutagenic, and so, it is reasonable to predict that the sensor for negative phototaxis can detect UV light thus minimising photodamage (Choi *et al*, 1999).

Mutagenesis studies in the thermophilic cyanobacterium, *Thermosynechococcus elongatus* suggest *cikA*, the gene encoding a phytochrome is the trigger for resetting the circadian clock to coincide with the day-night cycle (Schmitz *et al*, 2000). It has been observed that there are differences in gene expression depending on time of day that the samples were obtained.

Longer term adaptations to the light environment include Complementary Chromatic Adaptation (CCA), and responding to the spectral quality of the light and regulation of PSII/PSI, the latter response being to alterations in either spectral quality or intensity of the light (Grossman *et al*, 1993, for review).

Studies of CCA have focused on cyanobacterial species which possess both phycoerythrin and phycocyanin. While light of a 650 nm wavelength, characteristic of red light, will promote phycocyanin synthesis, a wavelength of 540 nm, green light, will favour an increase in transcription of phycoerythrin genes. A candidate gene for the sensor may be a phytochrome-like chromophore attached to a sensor kinase for the phycocyanin trigger, and a different chromophore for the response to green light. Mutagenesis of *Fremyella diplosiphon* indicates the involvement of a phospho-relay system in action (Grossman and Kehoe, 1997).

In *Synechocystis* 6803, it has been observed that the PSII/PSI ratio increases upon acclimation to high intensity light conditions owing to the suppression of PSI biosynthesis. Regulation of photosystem stoichiometry is conserved in phototrophic organisms, as, in addition to its observation in cyanobacteria (Fujita *et al*, 1987), similar behaviour has been recorded in red algae (Cunningham *et al*, 1990) and in higher plants (Melis and Harvey, 1981). This suggests that an organism capable of such regulation possesses an evolutionary advantage (Chow *et al*, 1990). Two known inactivation mutants of this regulatory system are *rppA*$^-$ (a sensory histidine kinase) (Li and Sherman, 2000) and *PmgA*$^-$ (Hihara *et al*, 1998). As yet, there is no data regarding whether each of these candidates for redox signal transduction proteins are implicated in the same signal transduction cascade. It has, however, been established that RppA is able to regulate the expression of photo-pigment genes by detecting alterations in the redox state of the plastoquinone pool.

1.5. Redox control of gene expression in photosynthetic organisms

1.5.1. Redox

Redox is a contracted term for reduction and oxidation. A molecule may be reduced if it gains hydrogen, loses oxygen or gains electron(s). The reverse is said to be true of oxidation. In biological systems, both forms of a molecule may co-exist, and under the principles of "redox control", may cause effects at a molecular level by acting as signalling molecules.

1.5.2. Redox control in photosynthetic organisms

The electron transport chain of electron carriers is an obvious candidate for research into improving the understanding of how gene expression is influenced by redox reactions (Pfannschmidt *et al*, 2001). As with many signalling pathways, a stimulus is detected by a receptor (or signal perceptor), and a cascade of reactions follows (signal transduction), finally effecting a response, such as the expression of a gene.

1.5.2.1. Key redox molecules

A variety of chemicals respond directly to certain wavelengths and intensities of illumination by either donating an electron (and becoming oxidised), or accepting one (thus becoming reduced). Amongst these, are haems, flavins, thiols and iron-sulphur centres, all of which are present in the photosynthetic apparatus of both prokaryotic and eukaryotic photoautotrophic organisms. In these organisms, to pinpoint the molecule that is critical to the signal, inhibitors of electron transport are employed. These include 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) (which inhibits electron transport through PSII) and 2,5-dibromo-3-methyl-6-isopropyl-1,4-benzoquinone (DBMIB) (an inhibitor of the Q_o site of the $cytb_6f$ complex). There have been several reports identifying the plastoquinone pool and/or the cytochrome bf complex as being the key signal perceptors, having effects on long term adaptations such as photosystem stoichiometry (Kim *et al*, 1993; Li and Sherman, 2000, for example) and the production of light harvesting complexes, as well as short-term ones like light state transitions (Mullineaux and Allen, 1990), in some cases, by activating enzymes such as kinases.

1.5.2.2. Types of redox control

Since redox reactions may be triggered by the presence (or absence) of light or oxygen, these two external environmental factors may provide a stimulus for redox control to occur. The type of response generated may be complex, so we may subdivide systems according to two types of redox control: perceptional and transductional. If the stimulus triggers a redox reaction in the sensory system, it is classified according to the first category, but if perception of the stimulus results in redox downstream, within a signal transduction molecule, then it is dubbed transductional control (Pfannschmidt *et al*, 2001).

1.5.2.3. Effect of oxygen on redox control in photosynthetic organisms

Not all prokaryotes capable of phototrophic growth are restricted to such mechanisms of growth. *Rhodobacter sphaeroides* 2.4.1 is one such species, capable of survival under diverse conditions. It is a purple bacterium capable of growth chemo and photoheterotrophically, as well as chemo and photolithotrophically. When external oxygen levels are below 3%, synthesis of photosynthetic genes is triggered. This is supposed to be catalysed by a cbb_3 cytocrome c oxidase forming a signal transduction pathway, where the amount of electron flow through the enzyme provides a signal perceived (Oh and Kaplan, 2000) and transmitted to a protein called PrrB possessing both kinase and phosphatase capability through a membrane-bound protein called PrrC. When oxygen levels are low, electron transport through cbb_3 oxidase is consequentially low, resulting in a repression of phosphatase activity, and an induction of kinase activity in PrrB. This produces a phosphorylated PrrA. This, in turn, results in the induction of photosynthetic gene expression. This state is considered to be the default pathway, with the repression of photosynthesis only induced when oxygen levels are higher (Oh *et al*, 2001).

1.5.2.4. Effect of light on redox control in photosynthetic organisms

1.5.2.4.1. Light intensity

There is a certain amount of evidence which points to an association between the intensity of light that cells are provided with and the redox molecule providing the signal to catalyse gene expression. Where illumination is sparse, the molecule in question is invariably plastoquinone (PQ), and it appears that its redox status is

involved in small regulations in order to maximise photosynthetic capability (Allen, 1985).

In higher plants, as light intensity increases, PQ becomes completely reduced, and so, fluctuations in the redox state of ferredoxin become important. Since the status of this intersystem electron carrier is dependent upon both cyclic and linear electron transport (unlike PQ, which is influenced by linear flow alone), the proportion of reduced and oxidised forms is more variable at intermediate light intensities. As illumination levels increase to a degree associated with photoinhibitory stress, thioredoxin is reduced and can no longer provide signals to trigger expression of genes crucial to responding to stress. Thus detection of the reduction of glutathione serves to transduce a response.

1.5.2.4.2. Wavelength

In higher plants, it has been shown that wavelengths of light transmitted to chloroplasts in different locations of a single leaf may be highly variable. Similarly, the crowding of unicellular photoautotrophs may also result in different conditions of light exposure. It is therefore of evolutionary importance that species possess mechanisms of maximising utilisation of light in these circumstances. It is predicted that such mechanisms are conserved in species capable of phototrophic growth, though we are some way from a comprehensive understanding of the signalling systems in action.

Some purple bacteria are motile via a photo-sensory transducer. In *Rhodospirillum centenum*, cells are positively phototactic towards infrared wavelengths, and negatively so with respect to wavelengths in the visible range (Ragatz *et al*, 1994 and 1995). Using mutants of the photo-sensory transducer gene, *ptr*, and inhibitors of the electron transport chain, it has been shown that Ptr senses the redox state of a molecule within the ETC and transmits a signal to induce a motility response (Jiang and Bauer, 2001).

According to data derived from mustard chloroplasts, the redox state of the PQ pool fine-tunes the PSII/PSI ratio by directly influencing the transcription of genes encoding subunits of both reaction centres (*psb*A and *psa*AB for PSII and PSI

respectively) within minutes of providing an illumination stimulus (Pfannschmidt *et al*, 1999). The results demonstrate that transcription of PSI subunits is increased in circumstances where PQ is reduced.

In cyanobacteria, it has been demonstrated that transcription of *psb*A genes is influenced by the status of the cytbf complex (Alfonso *et al*, 2000). In cyanobacteria, unlike in higher plants, there exists a family of *psb*A genes, which encode the D1 subunit of PSII, and these are differentially expressed according to the illumination wavelengths and intensities cells are provided with (Mohamed *et al*, 1993). Upon a switch from dark conditions to light, and therefore reduction of the PQ pool, *psb*A transcription is induced in *Synechocystis* 6803, and mRNA becomes destabilised. The stability of the mRNA transcripts is not considered to be affected by the illumination conditions itself, but by the redox state of the intersystem electron carriers. This was determined by using inhibitors of electron transport where cells remain illuminated. Under these conditions, mRNA transcripts of *psb*A remain stable (Alfonso *et al*, 2000).

1.6. State Transitions

1.6.1. Introduction

In phycobilisome-possessing micro-organisms, the state transition is a rapid adaptation mechanism in response to the light environment. It was first observed in the red alga *Porphiridium cruentum* by Murata (1969) and in the green alga *Chlorella pyrenoidosa* by Bonaventura and Myers (1969). Under conditions where light is preferentially absorbed by PSI, there will be characteristically a more efficient transfer of energy from the phycobilisomes to PSII. This is state 1.

It has been proposed that the mechanism has evolved in order to maximise excitation of the photosystems (Allen, 1992) but also to minimise photodamage (Schuster, 1986). The adaptation phenomenon has been observed in higher plants (Allen and Forsberg, 2001, for review) and is therefore conserved. However, there is evidence that the mechanism for achieving the state transition in plants and bacteria is different. In plants, there is strong evidence that the mechanism is linked

to phosphorylation. In cyanobacteria, state transitions have been proposed to be under the control of the redox state of an electron carrier in the plastoquinone pool, but the precise mechanism remains unknown here (Mullineaux and Emlyn-Jones, 2004).

1.6.2. The State Transition in Plants

Light-dependent kinase activity was first reported by Bennett in 1979. In plants, the state transition is mediated by phosphorylation of Light Harvesting Complex II (LHCII) by a kinase targeted to the thylakoid membrane. It has been shown that phosphorylation and dephosphorylation of LHCII is light-activated (Rintamaki *et al*, 1997; Hammer *et al*, 1997), but there is also evidence that some phosphatase activity is independent of both light and the redox state of the plastoquinone pool (Elich *et al*, 1997).

The kinase phosphorylates two subunits, Lhcb1 and Lhcb2 of LHCII, specifically at the site of the critical Thr-5 residue (Zer *et al*, 1999), when activated by the reduction of the plastoquinone pool. Plastoquinone molecules are reduced to plastoquinol molecules, which then associate with the Q_o site of cytochrome bf complex. The Q_o site must be bound in order to activate the kinase (Zito *et al*, 1999). The phosphorylation event mediated by LHCII kinase serves to dissociate LHCII from PSII, possibly owing to a conformational change in the phosphorylated complex, catalysed by the formation of an α-helix at the site of the phosphorylated threonine residue. This may consequently prevent binding to PSII (Nilsson *et al*, 1997). There is evidence to show that LHCII then associates with PSI (Allen and Forsberg, 2001), in addition to its dissociation from PSII. It is unknown how much of the phosphorylated LHCII associates with PSI. The LHCII kinase continues to be active provided that plastoquinol remains associated with the Q_o site of the cytochrome bf complex (Vener *et al*, 1997). *Chlamydomonas reinhardtii* mutants which do not possess cytbf, are incapable of activating the kinase, regardless of the redox status of plastoquinone, and so, are perpetually in state 1 (Lemaire *et al*, 1986; Wollman and Lemaire, 1988; Gal *et al*, 1990). Gal *et al* (1988) have reported

similar findings in higher plants. Figure 1.6 provides a summary of the proposed mechanism for the occurrence of eukaryotic state transitions.

The conditions for which phototrophic organisms bring about States 1 and 2 are apparently dependent upon the extent of electron flow into the PQ pool. In higher plants, there is more energy transfer to PSII, and the plastoquinone pool is essentially in an oxidised state in the dark, whereby LHCII is un-phosphorylated and is capable of binding to PSII. The kinase termed Stt7 in *Chlamydomonas* has been determined to be the enzyme principally involved in phosphorylation of LHCII, and hence, a key player in the transition to state 2 in photosynthetic unicellular eukaryotes (Depege *et al*, 2003). Its orthologue in *Arabidopsis thaliana* was recently identified, the inactivation mutant giving a state transition negative phenotype owing to its inability to phosphorylate LHCII (Bellafiore *et al*, 2005).

Conversely, in cyanobacteria, where oxidative phosphorylation for aerobic respiration is being carried out using the same electron transport chain as is used for photosynthesis in the thylakoids (Scherer, 1990), there are electrons being pumped into the plastoquinone pool while in the dark. Therefore, in the dark, a higher proportion of plastoquinone molecules can be in the reduced rather than their oxidised state (Mullineaux and Allen, 1986), and the transition to state 2 can be achieved by incubating cells in the dark (Mullineaux and Allen, 1990). In cyanobacteria, it is unknown whether the state transition is mediated by a reversible phosphorylation event, but it is controlled by the redox state of an electron carrier in the plastoquinone pool (Mullineaux and Allen, 1990).

1.6.3. The Cyanobacterial State Transition

1.6.3.1. Electron Transport

Various reagents have been used to elucidate the mechanism for the cyanobacterial state transition. Phenyl-1,4-benzoquinone (PBQ) and 2,5-dibromo-3-methyl-6-isopropyl-1,4-benzoquinone (DBMIB) are benzoquinone analogues, whose presence affects state transitions in this eubacterial phylum. It is proposed that this

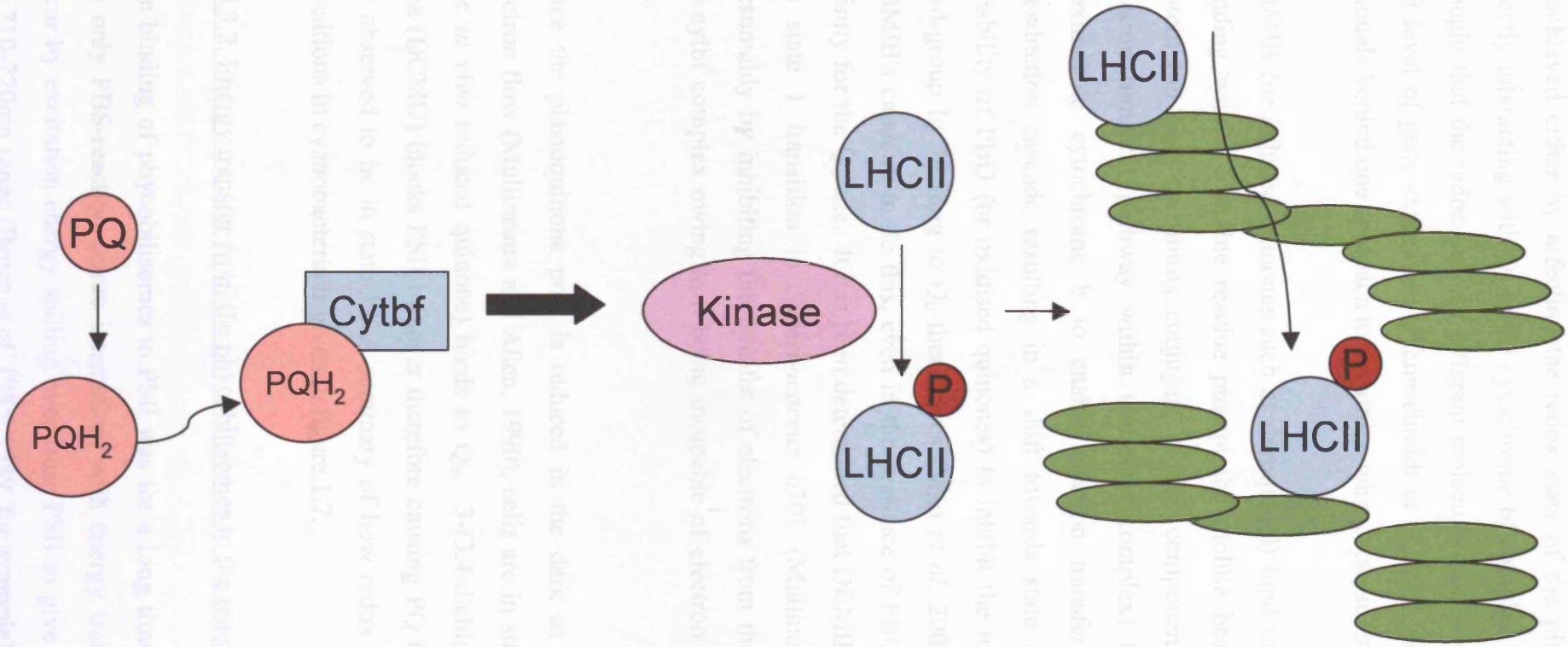

Figure 1.6. The state transition in plants. Plastoquinone (PQ) becomes reduced to plastoquinol (PQH2), which binds to the Qo site of the cytochrome bf complex, inducing the LHCII kinase. Phosphorylation of the LHCII results, and the complex now moves from the PSII-rich grana onto PSI centres found in stromal lamellae.

is achieved either by affecting the redox state of the plastoquinone pool or by directly interacting with Q_o in the cytochrome bf complex (Mao *et al*, 2002). It is thought that the redox state of different molecules can have a bearing on the type and level of gene expression (Pfannschmidt *et al*, 2001, for review). This is the rationale behind one approach to determine what catalyses state transitions.

DBMIB (or reduced quinones such as duroquinol) bind to Q_o and, by Hydrogen-bonding with a histidine residue prompt the mobile head-group of the Rieske protein (an Fe-S-containing conjugate protein component of the high potential electron transport pathway within the cytbf complex) to move to a position proximal to cytochrome b to enable electron transfer and trigger a signal transduction cascade resulting in a shift towards state 2. This overrides the capability of PBQ (or oxidised quinones) to inhibit the movement of the Rieske head-group by binding to Q_o themselves (Mao *et al*, 2002). The reason behind DBMIB's capacity to do this, even in the presence of PBQ is owing to its higher affinity for the Q_o site. It has been determined that DBMIB acts as an inhibitor of the state 1 transition in *Synechococcus* 6301 (Mullineaux and Allen, 1990) presumably by inhibiting the transfer of electrons from the plastoquinone pool to the cytbf complex owing to it being incapable of electron transfer when bound to Q_o.

Since the plastoquinone pool is reduced in the dark as a result of respiratory electron flow (Mullineaux and Allen, 1990), cells are in state 2 when plastoquinol (the *in vivo* reduced quinone) binds to Q_o. 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) blocks PSII turnover therefore causing PQ to be oxidised and cells are observed to be in state 1. A summary of how redox control influences state transitions in cyanobacteria is given in figure 1.7.

1.6.3.2. Energy transfer from the phycobilisomes to the reaction centres

The binding of phycobilisomes to PSII was for a long time widely accepted to be the only PBS-reaction centre interaction, with energy transfer to PSI thought to occur by excitation energy spilling over from PSII to give a characteristic peak in the 710-720nm range (Bruce *et al*, 1985; 1989, for example).

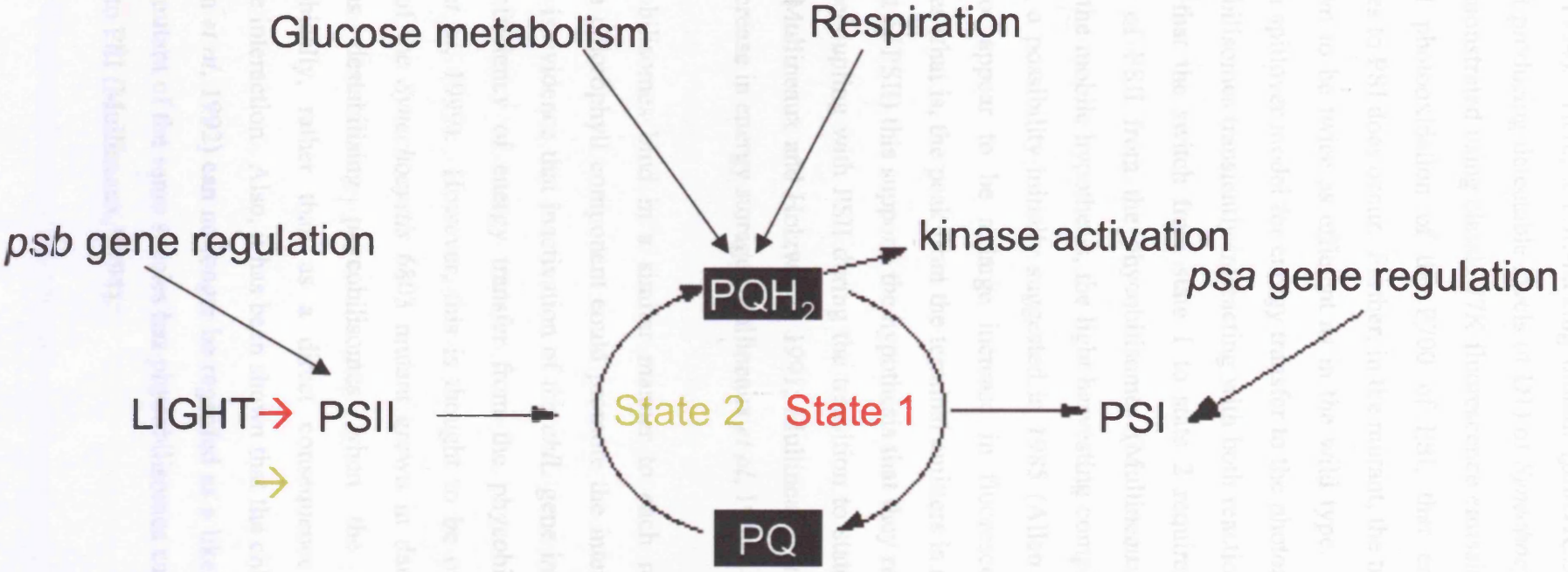

Figure 1.7. Summary of PQ modulated redox control in cyanobacteria.
Modified from: http://www.biology.purdue.edu/.../Impact2.html. Red illumination preferentially excites PSI, so more energy transfer occurs to PSII as a result of the plastoquinone pool being oxidised. Yellow light does the reverse.

However, energy transfer from phycobilisomes directly to PSI has been reported (Mullineaux, 1994). Here, a PSII-lacking mutant (*psb*D1CDII$^-$: lacking D2 and CP43, and not producing detectable levels of D1) of *Synechocystis* 6803 was used, and it was demonstrated using classic 77K fluorescence emission spectra as well as flash induced photooxidation of the P700 of PSI, that energy transfer from phycobilisomes to PSI does occur. Further, in the mutant, the transfer from PBSs to PSI was shown to be twice as efficient as in the wild type. This result was not indicative of a spillover model for energy transfer to the photosystems. Rather, the mobile phycobilisomes transiently interacting with both reaction centres is implied. It is thought that the switch from state 1 to state 2 requires the decoupling of roughly 60% of PSII from the phycobilisomes (Mullineaux and Allen, 1990). According to the mobile hypothesis, the light harvesting complexes would then re-attach to PSI, a possibility initially suggested in 1985 (Allen *et al*, 1985). Since there does not appear to be a large increase in fluorescence from unbound phycobilisomes (that is, the peak from the terminal emitters is not greatly increased relative to that of PSII) this supports the hypothesis that they reattach to PSI, rather than simply decoupling with PSII during the transition to state 2 (Mullineaux and Allen, 1990; Mullineaux and Holzwarth, 1991; Mullineaux *et al*, 1991). Further, there is no decrease in energy storage (Mullineaux *et al*, 1994).

Should phycobilisomes bind in a similar manner to each reaction centre, it is feasible that a chlorophyll component could promote the interaction (Mullineaux, 1992). There is evidence that inactivation of the *chl*L gene in *Synechocystis* 6803 reduces the efficiency of energy transfer from the phycobilisomes to reaction centres (Yu *et al*, 1999). However, this is thought to be owing to chlorophyll biosynthesis of the *Synechocystis* 6803 mutant grown in darkness being greatly reduced, thus destabilising phycobilisomes when the mutant is grown photoautotrophically, rather than as a direct consequence of a chlorophyll-phycobilisome interaction. Also, it has been shown that the chlorophyll component CP43 (Nilsson *et al*, 1992) can no longer be regarded as a likely candidate, since a PSII-lacking mutant of the same species has phycobilisomes capable of transferring energy direct to PSI (Mullineaux, 1994).

The PSII$^-$ mutant previously described is incapable of state transitions, even when an agent to oxidise the plastoquinone pool (thereby triggering a state 1 transition) is added. This provides strong evidence that PSII is required for state transitions to occur, and that the switch from one state to the other may be dependent upon a characteristic or event, which alters the affinity for PSII.

If a covalent modification, for example, a phosphorylation event (Allen *et al*, 1985; Allen, 1992) permits the phycobilisomes to dissociate from PSII, and therefore couple with PSI, but that interactions between phycobilisomes and PSI are less strong than those with PSII, it may explain why PBS-PSI interactions are more common in the mutant. There is a small proportion of functionally decoupled phycobilisomes in the PSII-free mutant, in spite of a lower phycobilisome to PSI ratio as compared with the wild type (Mullineaux, 1994).

The postulated covalent modification to prompt state transitions could occur in the reaction centres or in the phycobilisomes to sway the ratio in the type of phycobilisome-photosystem complex formed. RpaC-lacking mutants in *Synechocystis* 6803 (see 1.6.3.4) in a PSI-less or PSII-lacking background both show altered ability to transfer energy between the light harvesting complexes and the one remaining reaction centre (Emlyn-Jones *et al*, 1999). No differences in phospho-proteins in the thylakoid membrane could be found in a simple *rpa*C inactivation mutant when compared with wild type *Synechocystis* 6803, though this may simply be because RpaC is produced in very small quantities. Therefore, the possibility that phosphorylation is not involved cannot be discounted. Phosphorylation of linker proteins within the phycobilisomes of *Synechocystis* 6803 has been shown to be related to assembly and maintenance of phycobilisomes, and may be involved in signalling upon changing light environments (Piven *et al*, 2005).

The mechanism for how the phycobilisomes interact with the photosystems under different illumination conditions is the subject of controversy; two proposals at the forefront of this debate, mobile phycobilisomes and spill-over from PSII to PSI are discussed below.

1.6.3.3. The Mobile Phycobilisome Model versus the Spill-over Model (figure 1.8.)

It has been shown by using Fluorescence Recovery After Photobleaching (FRAP) that phycobilisomes are able to diffuse across the thylakoid membranes (see 1.8.3. for details). It is proposed that they are highly involved in the mechanism of the state transition where the degree to which each photosystem receives photons is determined by the wavelength and intensity of light present (Mullineaux *et al*, 1997). Phycobilisome diffusion is notably much faster than the observed diffusion rates of light harvesting complexes of green plants (Drepper *et al*, 1993).

The spill-over model for the state transition in cyanobacteria is that the phycobilisomes are permanently associated with PSII and that excitation energy spills over to PSI during state 2 giving the observed fluorescence increase of PSI (Biggins and Bruce, 1989). The findings of Olive *et al* (1986) support this theory. Freeze fracture experiments on thylakoid membranes suggest that PSII complexes are arranged in rows during state 1, but during the transition to state 2, randomisation occurs, leaving them in closer proximity to PSI. This would allow for excitation energy to spill over into PSI during state 2. Further, that phosphorylation events causing PSII to become more negatively charged would promote these exoplasmic face (EF) particles to repel each other, which suggests why the randomisation event could occur.

The capability for PSII particles to move is in conflict with evidence from FRAP studies which show PSII to be immobile (Mullineaux *et al*, 1997). It is notable that freeze fracture studies cover short distances, whereas FRAP provides evidence for movement across large distances, so it is conceivable that both observations hold true.

Treating cells with high phosphate buffer following their acclimation to one state or the other traps the cells in that state (Mullineaux, 1993). The observation holds true for both fixation of state 1 and state 2, which may lend weight to the mobile model for the state transition, where a proportion of the phycobilisomes couple preferentially to one photosystem or the other in different light environments (Mullineaux, 1992). Phycobilisome diffusion has been observed using the FRAP

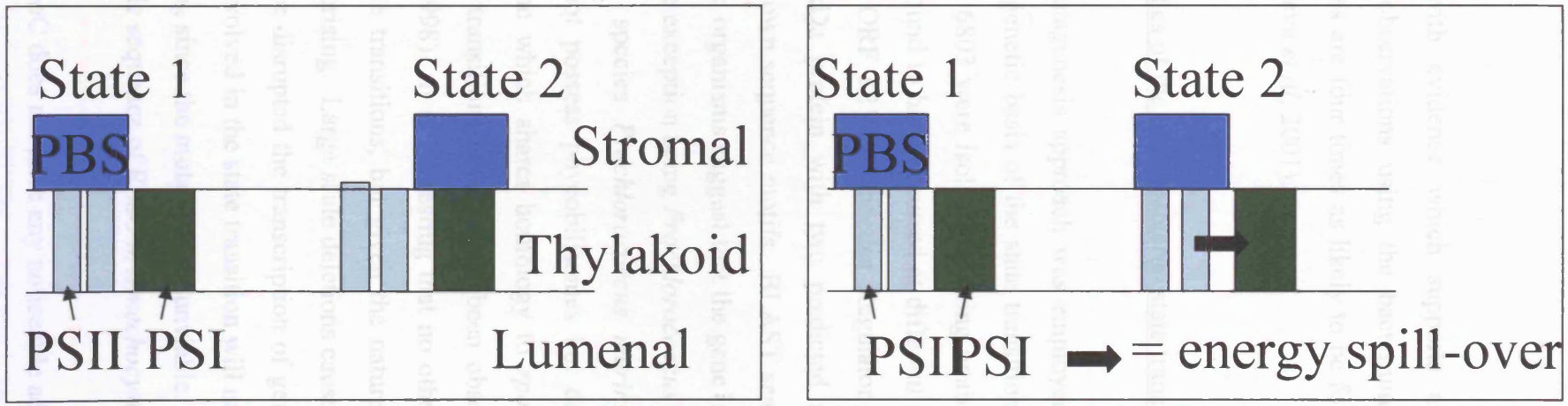

Figure 1.8. Mobile model (left) versus Spill-over model (right) for state transitions

technique, which supports the mobile model for the state transition (Mullineaux *et al*, 1997).

In conflict with evidence which supports a spill-over mechanism for state transitions, observations using the bacterium *Spirulina platensis* suggest that phycobilisomes are four times as likely to be found interacting with PSI than PSII (Rakhimberdieva *et al*, 2001).

1.6.3.4. Genetics of the cyanobacterial state transition

A random mutagenesis approach was employed by Emlyn-Jones *et al* (1999) to elucidate the genetic basis of the state transition in cyanobacteria. Six mutants in *Synechocystis* 6803 were isolated as being unable to perform state transitions, and all six were found to have occurred at different sites within the same open reading frame. The ORF sll1926 encodes Regulator of Phycobilisome Association C (RpaC), a 9kDa protein with two predicted membrane-spanning domains and bearing no known sequence motifs. BLAST searches within genome sequences of photosynthetic organisms suggest that the gene is conserved in most cyanobacterial organisms, the exception being *Prochlorococcus marinus* MED4, a high-light living strain of the species *Prochlorococcus marinus*. Curiously, *Prochlorococcus* bacteria do not possess phycobilisomes but do (with the exception of MED4) possess a gene which shares homology to *rpa*C (Mullineaux and Emlyn-Jones, 2004). State transitions have indeed been observed in this species too (van der Staay *et al*, 1998). It is interesting that no other genes were discovered as being crucial to state transitions, but given the nature of the method employed to find them, not surprising. Large scale deletions caused by the approach will have either knocked out or disrupted the transcription of genes crucial to cell viability; hence, other genes involved in the state transition will not have been categorised if close to essential genes, since the mutants were unviable. Figure 1.9 shows the alignment of the polypeptide sequence of RpaC in *Synechocystis* 6803 and *Synechococcus* 7942.

Deletion of *rpa*C does not have any noticeable adverse effects on capability to carry out general biochemical processes within the cell. Mutants of this kind are

Figure 1.9. Amino acid sequence alignment of RpaC in *Synechocystis* 6803 (top) and *Synechococcus* 7942 (bottom). Both have two transmembrane helices (in the boxes), but the putative protein of *Synechococcus* 7942 has a C-terminal extension not present in *Synechocystis* 6803.

permanently in state 1, whereby the phycobilisomes are associated with PSII rather than PSI according to the mobile model for the state transition. Potentially, there could be a small amount of spill-over of excitation energy from PSII to PSI, though this would not be predicted if the mobile model was the sole basis for the state transition. If the physiological role of the state transition is to optimise conditions for absorption of light energy and therefore photosynthetic capability, then mutants trapped in state 1 are still optimised to light harvesting in red light conditions. They perform as well as wild type cells when provided with chlorophyll-absorbed red-wavelength illumination. The mutants are only at a slight disadvantage when cultured in low intensity yellow light absorbed by phycocyanin, as the phycobilisomes are not transferring sufficient photons to PSI chlorophyll molecules. Therefore, it is not surprising that it is only under these conditions the mutant cells have slower replication rates.

Since the phenomenon of the state transition is only observed in cells grown at relatively low light intensities (Emlyn-Jones *et al*, 1999), *rpa*C transcript levels are only detectable at these low light levels in wild-type cells (Hihara *et al*, 2001), and that the knock-out mutant is not more sensitive to photoinhibition (Emlyn-Jones, PhD thesis, 2000), it is unlikely that the action of RpaC serves to reduce photodamage. Consequently, it is unlikely that the state transition is evolutionarily conserved as a protective measure to such damage.

1.6.3.4a. Other mutants which potentially inform the mechanism of state transitions

It is widely accepted that the redox status of the plastoquinone pool (Mullineaux and Allen, 1986) or that of the cytochrome bf complex (Vernotte *et al*, 1990) is the trigger for inducing a state transition. As such, it is necessitated that mutations which affect the redox status either directly or indirectly will help to inform the mechanism of state transitions. Unfortunately, since Cytbf is required for cell viability in cyanobacteria (owing to its respiratory requirement in addition to its contribution to the photosynthetic apparatus), an inactivation mutant in these prokaryotes is unattainable. However, mutants of both phycobilisomes and NADPH dehydrogenase have been acquired.

First, a mutation was made within the APC-B subunit of the phycobilisome of *Synechococcus* PCC 7002 and has been termed *apc*D$^-$. The gene encodes a small part of the allophycocyanin core of the phycobilisome and corresponds to a terminal emitter of fluorescence (Zhao, 1992). A similar mutant has been made in *Synechocystis* PCC 6803 (Ashby and Mullineaux, 1999a). Additionally, an *apc*F$^-$ mutant (deficient in APC-B$^{18.5}$ subunit) was made and is also incapable of state transitions. These mutants may be incapable of state transitions on account of altered energy transfer from the phycobilisomes to photosystems, and observations suggest that the APC-B$^{18.5}$ subunit is more critical to the issue than the subunit encoded by *apc*D (Ashby and Mullineaux, 1999b).

While these data suggest that the phycobilisome core plays a critical role in the potential for *Synechococcus* sp. PCC 7002 to carry out state transitions, deletion of *cpc*BA genes required for the synthesis of the phycocyanin peripheral rods of the hemidiscoidal light-harvesting complexes does not inhibit either the ability to make light-state transitions or alter the kinetics thereof (Zhao *et al*, 2001). The observation that these mutants display state transitions of smaller amplitude to wild type cells may be explained by the smaller absorption cross-section by the rod-lacking phycobilisomes. Compensating for this, and the consequently decreased energy transfer to PSII, there is approximately twice the quantity of PSII in the mutant compared with the wild type (Zhao *et al*, 2001).

An NADPH dehydrogenase inactivation mutant, *ndh*B$^-$, exhibits significantly reduced cyclic electron transport around PSI. It is a mutant perpetually locked in state 1, since the PQ pool remains oxidised (as the dehydrogenase functions to reduce it). The same applies to the cytbf complex (Schreiber *et al*, 1995).

It is significant that state transitions are known to occur in phycobilisome-less cyanobacteria. *apc*A$^-$, and the double mutant *apc*A$^-$/*apc*B$^-$ which lack the PBS core and the whole PBS respectively are capable of generating state transitions. Bruce *et al* (1989) worked on the *Synechococcus* 7002 double mutant and found that state 1 could be induced by exciting chlorophyll molecules of PSI with a long wavelength of light. State 2 applied if cells were adapted using a shorter wavelength. This research suggests that the phycobilisomes themselves are not crucial to the state

transition phenomenon. Su *et al* (1992) in studies of the *apc*A$^-$ mutant found that the fluorescence of PSII was lower than in the wild type cells, but that there were no significant differences for PSI. Since the PSI excitation spectrum was approximately equivalent to the phycocyanin absorption spectrum, it was concluded that the energy transfer from phycocyanin to PSI is critical to inducing state transitions.

It has been reported, in two separate organisms, *Synechococcus* 7002 and *Synechococcus* 7942, that deletion of *psa*L, the gene encoding the polypeptide subunit which facilitates the formation of PSI trimers is accompanied by a faster rate of state transitions as well as faster phycobilisome diffusion with no other observable differences in phenotype than that of wild-type cells (save for slightly slower growth rates with blue light illumination) (Schluchter *et al*, 1996 and Aspinwall *et al*, 2004). That is to say, that in these mutant organisms, which possess just monomeric PSI, the transition from state 2 to state 1 is faster. Are the phycobilisomes able to dissociate better from the monomeric form and so, to couple to PSII more readily? In order to elucidate what is really occurring, it is necessary to determine the rate of the transition from state 1 to state 2. Should state transitions also be significantly faster than that of wild-type cells, more efficient dissociation of phycobilisomes from PSI may not be the critical explanation for observable differences, since transition from state 1 to state 2 would require the light harvesting apparatus to associate with monomeric PSI. It may lend further weight to the theory that state transitions are speeded simply on account of the smaller complexes in the membrane promoting easier movement of the phycobilisomes between PSI and PSII. It is, however, technically difficult to extract a measurement of speed of the transition from state 1 to state 2.

Perhaps there is a greater requirement for the state transition in the mutant cells whereby monomers are of lower capability to promote optimal photosynthesis, so the state transition is up-regulated to compensate for the shortfall? Since it is also known that RpaC expression is a requirement for state transitions in *Synechocystis* 6803, is it possible to detect higher levels of expression in the *Synechococcus* 7942 homologue of RpaC in these mutants?

1.6.5. The Role of State Transitions in Cyanobacteria

There are three main proposed functions of the state transition in cyanobacteria. First, and most likely, is that the state transition serves to maximise the potential for most efficient light harvesting, and therefore photosynthetic capability. Next, the phenomenon would increase ATP stores by augmenting the amount of cyclic photophosphorylation in the cell when ATP is low. The final proposal is that state transitions minimise photodamage to the photosystems.

1.6.5.1. Maximising light harvesting

State transitions are induced in response to different wavelengths of light and under low illumination conditions in order to make most efficient use of light available by balancing the turnover of PSI and PSII (Murata, 1969; Emlyn-Jones *et al*, 1999 and others).

1.6.5.2. Increasing ATP content in cells

There are two types of electron flow within the thylakoid membranes. Linear electron flow describes the condition where electrons are passed from PSII to PSI via the cytbf complex, resulting in $NADP^+$ being reduced to NADPH. The other, cyclic flow, generates ATP but not NADPH, since electrons reaching PSI are passed through the PQ pool and the cytbf complex and into a cycle around proteins within PSI thereby generating the electrochemical gradient enabling a proton pump to generate ATP.

Linear electron transport generates ATP and NADPH in roughly the correct quantities for use in the Calvin cycle. However, since ATP is used for many other biochemical processes in the cell, ATP can become depleted. Linear electron transport is therefore not sufficient to provide the correct ratio of ATP/NADPH (Allen, 2004). One way of counteracting this would be to minimise use of PSII and maximise cyclic electron flow around PSI. This is effectively the transition to state 2.

So, one possible explanation for the existence of cyclic photophosphorylation is to generate sufficient ATP to compensate for the shortfall from linear photophosphorylation (Bulte *et al*, 1990; Vallon, 1991). The observation in *Chlamydomonas* that there are between 14% and 20% more chlorophyll molecules in PSI than in PSII could be critical if this is indeed the reason for its evolution in eukaryotes. It is important to note that, as yet, experiments have mainly been conducted on eukaryotic organisms to investigate the effect of ATP depletion on state transitions. The ratio of chlorophyll molecules in PSI relative to PSII is far higher in cyanobacteria, suggesting that cyclic flow could be more important, but since cyanobacteria have phycobilisomes, it is difficult to compare the prokaryotes with the eukaryotes.

Cyclic photophoshorylation requires that some of the components are oxidised and some are reduced. If all were oxidised, there would be no electrons to cycle around PSI, and if all were reduced, there would be nowhere to transfer electrons to. Each electron carrier must therefore act as both a donor and an acceptor. In *Synechocystis* 6803, NAD(P)H dehydrogenase is thought to be key component in the induction of cyclic photophosphorylation (Mi *et al*, 1992, 1994). This enzyme oxidises NADPH, and so generates electrons, which can cycle around PSI in order to augment ATP levels (Fork and Herbert, 1993).

The other potential function of the state transitions, linked to the above point would be to maintain the ATP/NADPH ratio given by linear electron transport (Turpin and Bruce, 1990; Vallon, 1991).

1.6.5.3. Protective function

Photoinhibition, that is, the inactivation of photosynthetic electron flow as a result of excessive exposure to high light intensity is a phenomenon that was first described by Jones and Kok (1966a, 1966b). It has been established and extensively discussed (see Keren and Ohad, 1998 for review), that photosystem II is susceptible to photodamage, whereby its major component, the D1 protein is

degraded. While most studies have focussed on the unicellular eukaryote, *Chlamydomonas reinhardtii* (Ohad *et al*, 1984, for example), observations in *Synechococcus* sp. PCC 6301 suggest a protective function for the state transition. Under conditions of prolonged exposure to high light intensities, cells make a transition from state 1 to state 2, thus increasing energy transfer to PSI, supposedly minimising the potential for damage to the more susceptible photosystem (Rouag and Dominy, 1994). Since an alternate opinion of state transitions functioning to maximise use of the photosynthetic light harvesting apparatus and energy transfer under minimal light intensities, this theory is also the subject of debate. However, it is supported by observations of cyanobacterial blooms whereby state 1 is induced as usual by illumination which includes red light, state 2 is facilitated in deeper water where it is far darker and interestingly, a state 2 transition is recorded when cells are illuminated with bright white light in this *in situ* study (Schubert *et al*, 1995). The fact that the *Synechocystis* 6803 *rpaC*$^-$ mutant is no more susceptible to photoinhibition than the wild type does not support the theory that state transitions have a photo-protective function (Mullineaux and Emlyn-Jones, 2004).

1.7. This thesis: Phycobilisomes and the cyanobacterial state transition

The rationale behind the project is to generate clues as to the mechanism of the state transition in cyanobacteria. One of the primary objectives is to determine where the protein RpaC is targeted. Given that it has two putative membrane-spanning domains, it needs to be established whether the protein is targeted to the plasma membrane or the thylakoid membrane. Once this has been elucidated, realising what complexes RpaC interacts with will be of importance.

If a GFP-tagged RpaC could be produced in *Synechocystis* 6803, not only would this highlight where the protein is targeted, it would also enable us to distinguish whether the protein is localised to different regions of a cell depending on whether the cell has been adapted to state 1 (red light) or state 2 (low light). Further, if the distribution of phycobilisomes in each of the two states is different, and the distribution of the GFP overlaps with this, it may suggest that RpaC is associating with the phycobilisomes, which have previously been determined to distribute light to preferentially to one of the two photosynthetic complexes depending on the

illumination conditions. As *rpa*C mRNA is not detectable in wildtype, an over-expressor was generated, and future GFP work might exploit this.

It is not possible to do Fluorescence Recovery After Photobleaching (FRAP) (See 1.7.3 for details) experiments with *Synechocystis*; however, *Synechococcus* 7942 lends itself well to such research, complete genome sequences being readily available, enabling easy generation of mutants of interest.

1.8. Key experimental techniques

1.8.1. Molecular biology

With the advent of automated DNA sequencing, it is becoming routine to generate mutants in cyanobacterial species. Several species have already been completely sequenced, including those featured in this thesis, *Synechocystis* 6803 (Kaneko and Tabata, 1997), *Synechococcus* 7942 (http://genome.jgi-psf.org/draft_microbes/synel/synel.home.html). Further, some, including these species, are naturally transformable, taking up exogenous DNA with relative ease. Inactivation constructs and over-expression mutants are the focus of much of the work presented here.

1.8.2. Fluorescence Spectroscopy

State transitions were first observed in the red alga, *Porphyridium cruentum*, with 77K fluorescence emission spectra of samples frozen in liquid nitrogen. Murata (1969) noted the difference in relative fluorescence from the reaction centres in the cells depending on the wavelength of light they were excited with prior to being frozen. The phenomenon of the state transition was observed in a cyanobacterium, *Synechococcus lividus* by Fork and Satoh (1983). Under illumination conditions where light is preferentially absorbed by PSI, the amount of fluorescence from PSII is observed to increase. This is observed whether exciting phycobilins (at 600 nm) or chlorophyll (at 435 nm), thus exhibiting a larger emission peak at 685 and 695 nm as compared with the fluorescence observed when given illumination at wavelengths typically absorbed by PSII relative to fluorescence of PSI, at 720nm (Murata, 1969). The emission peak at 695 nm corresponds to fluorescence

primarily from PSII, the peak at 685 nm being attributable to a combination of chlorophyll from PSII and the terminal emitters of the phycobilisomes. Much of the chlorophyll in cyanobacterial cells is affiliated with PSI, and this is highly evident when comparing the intensity of emission peaks corresponding to the two reaction centres upon excitation at 435 nm.

1.8.3. Fluorescence Recovery after Photobleaching (FRAP)

The dynamics of photosynthetic membranes in autotrophic prokaryotes may be studied using this technique, particularly as in certain species where there is a relatively uniform distribution of components within the membrane. It permits the observation of mobility of fluorophore-tagged biological molecules, including photosynthetic pigments such as chlorophyll (Mullineaux *et al*, 1997; Sarcina *et al*, 2001) and phycobiliproteins (Mullineaux *et al*, 1997) (see figure 1.10, for example). Additionally, proteins synthetically tagged with GFP (Reits and Neefjes, 2001), for example, may also be observed in motion. In some cyanobacteria, *Synechococcus* 7942, for example, it is possible to accurately determine the rate of diffusion of such fluorescent molecules.

FRAP measurements may be obtained using a laser scanning confocal electron microscope (Kubitscheck *et al*, 1994). By increasing the laser power for a short time (1-2 seconds), the fluorescent pigment being analysed will be bleached out in the region where the laser has scanned, resulting in this area producing no fluorescence. The laser power is then reduced, and scanning of the cell will occur to generate a finite number of images over a known time interval. Should the pigment be mobile, fluorescence will return to the bleached region over this period of time, and an estimate of the diffusion coefficient is obtainable provided that the membrane environment is similar across the majority of the cell whose dimensions are known.

By bleaching a line through an individual cell and observing for the recovery of fluorescence at a wavelength appropriate for the emission wavelength of GFP fluorescence, it is possible to resolve whether the tagged protein diffuses in a predictable manner. It is important to stress the point that, as yet, there have been

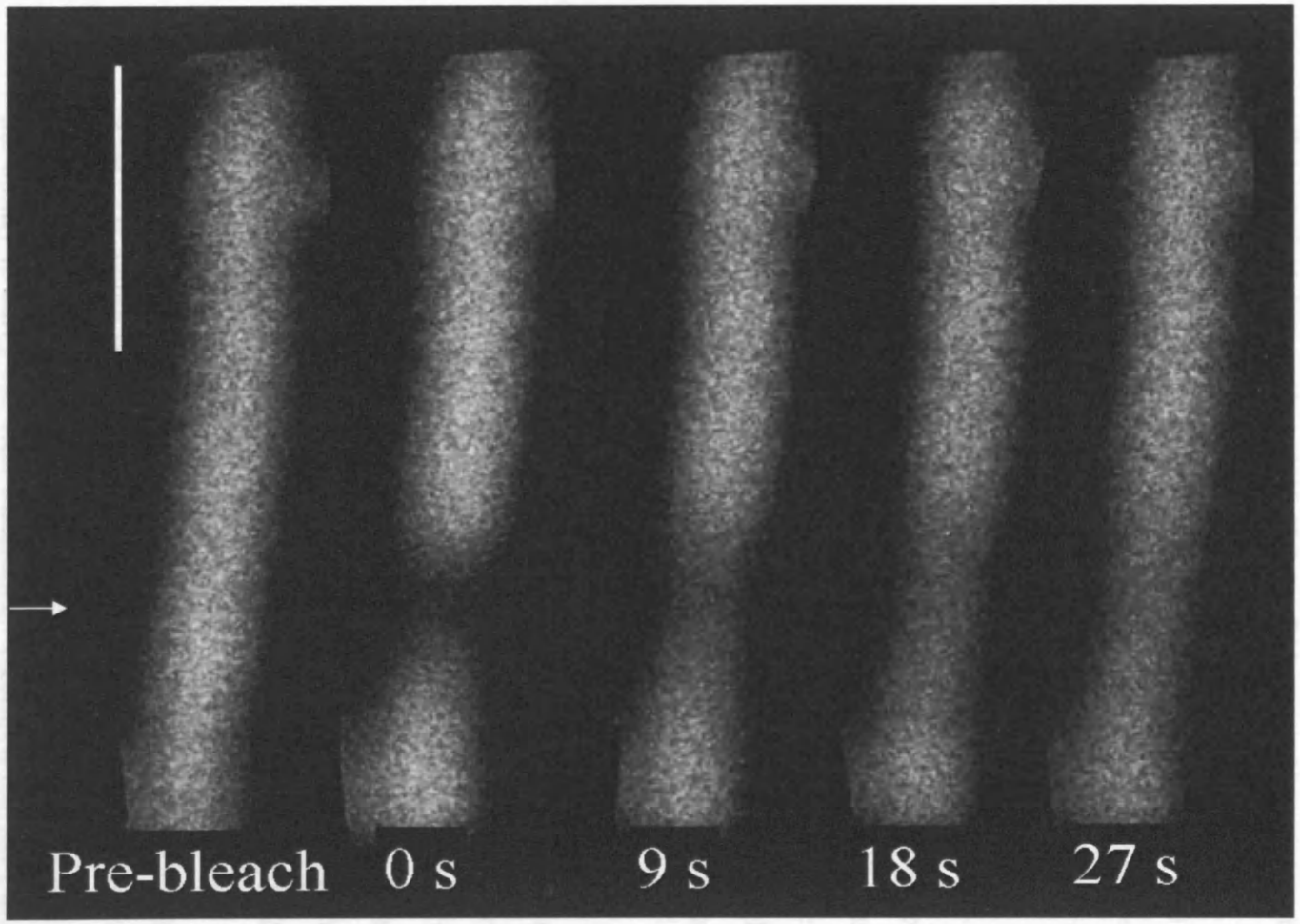

Figure 1.10. FRAP showing diffusion of phycobilisomes in *Synechococcus* 7942 in growth medium. Scale bar is 5μm. Arrow denotes where the bleach will be most intense. Taken from Joshua and Mullineaux, 2004.

no successful attempts to express GFP in *T. elongatus* or *Synechococcus* 7942. An *rpa*C inactivation mutant will be sought in an elongated strain. FRAP studies of such a mutant may suggest whether RpaC plays a role in phycobilisome mobility, and since GFP fluorescence does not overlap with that of chlorophyll or phycobilisomes (Spence *et al*, 2003), it may be possible to analyse the behaviour of all three components though to play a role in cyanobacterial state transitions.

PSII is known to be immobile (Sarcina *et al*, 2001) except if pre-treated with high intensity red light (Sarcina, personal communication). For a tagged protein which associates solely with this complex, an observation of marked diffusion would not be expected. The diffusion of the phycobilisomes (mobile model) across the thylakoid membranes from one photosystem to the other has been the subject of controversy. Some believe that the phycobilisomes are permanently associated with PSII (that is, do not diffuse), and that spill-over of excitation energy to PSI occurs when cells are dark adapted in order to optimise photochemical turnover. Either way, it is proposed that the transition to state 2 occurs because PSI turns over more efficiently than PSII, so in very dim light cells would be advantage if they could maximise the harvesting and distribution of light energy.

Transcription of *rpa*C in *Synechocystis* 6803 is detectable under low light conditions and in small quantity (Hihara *et al*, 2001). Therefore, it may be difficult to detect expression of a GFP-tagged version of the protein. It follows, therefore, that over-expression of RpaC may be required in order to visualise fluorescence of GFP in the cells. It is proposed that if over-expressing RpaC can be achieved without obscuring the state transition phenotype, a GFP-tagged protein may be sought by cloning the gene immediately downstream of the *psb*A2 constitutive promoter (Lagarde, *et al*, 2000) as PsbA2 expression is far greater than that of RpaC. It would be interesting to determine whether over-expressing RpaC results in faster state transitions, more efficient state transitions, or has a negative effect on the ability to carry out state transitions. Research carried out on a cyanobacterial mutant with solely monomeric PSI (rather than the wild-type mixture of trimeric and monomeric PSI complexes) has provided evidence that state transition speed and efficiency are amplified when PSI is monomeric. Over-expression studies may provide clues as to whether RpaC acts on PSI.

1.8.4. Atomic Force Microscopy

There have been several developments recently which aid the study of biological membranes. One such technique that has been optimised for such study is that of atomic force microscopy (AFM) (Binnig *et al*, 1986) and its derivatives (Hansma and Pietrasanta, 1998) which can be utilised effectively to provide information about the spatial topography of thylakoid membranes, the height of embedded molecules within them and the ultrastructure of these complex molecules, frequently proteins (See Hoh *et al*, 1992, for review).

The technique relies upon a stylus scanning over a sample and detecting elevations in the membrane by virtue of how the stylus tip interacts or repels the sample.

AFM has already been used to complement electron microscopy and X-ray crystallography studies to resolve the distribution of photosystems in wild type and PSI-mutant eukaryotes and prokaryotes (Kaftan *et al*, 2002 and Fotiadis *et al*, 1998 respectively). For example, understanding of the complex interaction between DNA and *E. coli* RNA polymerase has been aided by scanning force microscopy (Kasas *et al*, 1997; Rippe *et al*, 1997; Rivetti *et al*, 1999). Low temperature imaging of smooth muscle myosin has also been achieved (Zhang *et al*, 1997), and there has been a great deal of success in imaging proteins at extremely high resolution (Engel *et al*, 1999; Muller *et al*, 1998; Muller and Engel, 1999; Muller *et al*, 1999a).

Significantly, AFM technology is contributing to improved understanding of human diseases. Ataxia telangiectasia is a disorder which results in cancer predisposition and chromosomal instability resulting from the absence of a protein functioning to signal DNA damage to tumour suppressor genes at cell cycle control points. AFM has shown that the relevant protein, ATM, normally acts to bind DNA, but also to phosphorylate p53, a cell division suppressor, when DNA has been judged to be damaged (Smith *et al*, 1999). Much progress has been made in Alzheimer's research, where microscopy has been used to determine the structure of

lithostathine protofibrils (Gregoire *et al*, 2001) and the amyloid-β peptides (Stine *et al*, 1996) which contribute to neural plaques and consequently impairing neural function. Interaction of these proteins has also been studied extensively by exploiting this technique (Kowalewski and Holtzman, 1999; Yip *et al*, 2002).

There are, of course, limitations to the application of atomic force microscopy to biological membranes. The fact that the membranes are a relatively soft surface inhibits the ability of the stylus to scan across the surface without disturbing the contents. Improvements are being made in the technology to minimise such problems. Much of the early work has been using the purple membranes of the prokaryote *Halobacterium halobium* (Muller *et al*, 1995, for example).

In cyanobacteria, the phycobilisomes distribute light to the photosynthetic reaction centres embedded in the thylakoid membrane. Since it is, in theory, possible to detect to a resolution of 10 pm with AFM, large complex structures like PBSs should be easily detectable.

The debate surrounding state transitions in cyanobacteria revolves around how the PBSs interact with the photosystems. Evidence from fluorescence studies suggests that they interact in a highly transient manner, attaching and detaching according to the illumination conditions to which the cell is exposed (Mullineaux *et al*, 1997, for example). It is also known that PSII is immobile (Mullineaux *et al*, 1997; Sarcina *et al*, 2001). The postulates of the mobile model for state transitions are that when cells are provided with light preferentially absorbed by PSI, a greater proportion of excitation energy is channelled towards PSII. This is termed the state 1, where it is thought that the phycobilisomes are preferentially associating with PSII. The reverse has been proposed for the transition to state 2 (Allen *et al*, 1985, for example). The controversy surrounds whether the phycobilisomes are perpetually associating with PSII, and during the transition to state 2 a certain amount of energy spills over to PSI. AFM should provide valuable data in the quest for resolving this issue.

It is possible to isolate thylakoid membranes with functionally coupled phycobilisomes using SPCM buffer (Gantt, 1988). It has also been shown that

this buffer is capable of fixing cyanobacterial state transitions (and preventing re-adaptation to the alternate state) (Mullineaux, 1993). Therefore, membranes with phycobilisomes attached according to a state 1 conformation can be compared with those in a state 2 arrangement. A state transition-less mutant can be used, which would hopefully provide information as to how the absent protein is acting. Owing to the fact that RpaC is such a small, lowly expressed protein, it is not likely to be detected amongst all the other membrane proteins in the background with this technique.

Chapter 2: Materials and Methods

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2.1. Bacterial Strains and Growth Conditions

Wild type *Synechocystis* sp. (glucose tolerant) 6803 (donated by W. Vermaas, Arizona State University and characterised by Williams, 1988), *Synechocystis* sp. PCC 6803 (from the Pasteur Culture Collection and donated by N. Mann, Warwick University) and *Synechococcus* sp. PCC 7942 (from the Pasteur Culture Collection) cells were grown on BG11 liquid medium according to the recipe of Castenholz (1988). The media were supplemented with 10 mM TES buffer (pH 8.2), 0.121 M sodium thiosulphate and 10 mM $NaHCO_3$ at 30°C in an illuminated shaking incubator (New Brunswick). They were also maintained on BG11-1.5% agar plates in a stationary incubator.

*rpa*C$^-$ mutants of *Synechocystis* 6803 and *Synechococcus* 7942 were supplemented with 50 μg/ml kanamycin. The *rpa*C^{-++} mutant generated in *Synechocystis* 6803 was supplemented with 50μg/ml spectinomycin in addition to 50 μg/ml kanamycin, while the *Synechocystis* 6803 *rpa*C^{++} mutant was selected solely using 50 μg/ml spectinomycin. *Synechococcus* 7942Δrod was grown in BG11 with 5 μg/ml chloramphenicol.

Cell cultures were grown in white light unless otherwise specified. Light intensities of 70 $\mu mol.m^{-2}.s^{-1}$ (high), 40 $\mu mol.m^{-2}.s^{-1}$ (moderate) and <10 $\mu mol.m^{-2}.s^{-1}$ (low) were used depending on the experimental requirements.

For the purposes of iron starvation, *Synechocystis* 6803 cells were grown in moderate light in BG11 medium and transferred to BG11 free of iron components. They were transferred to fresh medium every 4 days for a period of 20 days until maximal fluorescence quenching capability had been reached.

For cyanobacterial growth experiments, the optical density at 750 nm of cell cultures was recorded over time. Cultures were started at an optical density of 0.1 and followed from lag phase to stationary phase. Tangents were drawn to the resultant growth curves at exponential growth phase, and the doubling times determined. Cultures were grown in triplicate, and standard deviations recorded.

Escherichia coli DH5α cells used for transformation experiments to generate mutants of *Synechococcus* 7942 and *Synechocystis* 6803 were grown on LB medium (Sambrook *et al*, 1989). Where appropriate and for selection purposes, the media were supplemented with ampicillin (100 μg/ml), X-gal (2%), IPTG (10 mM) and kanamycin (50 μg/ml) and/or spectinomycin (50 μg/ml).

2.2. Isolation of Nucleic Acids

2.2.1. DNA

2.2.1.1. Genomic DNA

Cyanobacterial cells (1ml minimum volume) suspended to an optical density of 2.0 at 750 nm (obtained using a Unicam Spectrophotometer (Cambridge, U.K.) were centrifuged for 15 minutes at 4500 rpm using an MSE Mistral 1000 centrifuge (Thermo Life Sciences, Hampshire, UK). The pellet of cells was re-suspended in 400 μl of TES buffer (5 mM Tris, pH 8.5, 50 mM NaCl, 5 mM EDTA) and incubated at 37°C in 100 μl of lysozyme (50 mg/ml), shaking occasionally. Following this, 50 μl of 10% sarkosyl was added and 600 μl phenol. The suspension was vortexed vigorously once a minute for ten minutes and centrifuged for 3 minutes in a microfuge. To remove RNA contaminants, RNase A was added to the supernatant and incubated at 37°C for 15 minutes. In order to remove the RNase, 100 μl of 5 M NaCl, 100 μl CTAB-NaCl (10% CTAB, 700 mM NaCl) and 600μl chloroform was added and vortexed once a minute for ten minutes. The supernatant from a 5 minute centrifugation step was added to 700 μl cold isopropanol, and the DNA pelleted by 15 minute centrifugation at 4°C. After rinsing and air-drying the pellet in 70% ethanol, the DNA was dissolved in TE buffer (Sambrook *et al*, 1989).

2.2.1.2. Plasmid DNA

The QIAprep Spin Mini-prep Kit was used, following the protocol outlined by the manufacturers (See also Sambrook *et al*, 1989). DNA was eluted in the Tris buffer provided, except where DNA was to be subsequently sequenced, when it was eluted in water.

2.2.2. RNA

Total RNA was harvested from cyanobacterial cells (5ml minimum volume) in exponential growth phase at OD750 of approximately 1.0. Cell pellets were re-suspended in twice the volume of RNA Protect reagent (Qiagen) and broken using 100 µl of lysozyme (50 mg/ml) for 20 minutes at room temperature. RNA minikit (Qiagen) was then used according to the manufacturer's instructions to purify RNA from the cell debris. An on-column DNaseI digestion (1 unit per 2 µg total RNA) treatment was undertaken for 15 minutes at room temperature (Qiagen) to remove any DNA impurities. RNA was finally eluted in 30 µl RNase free water.

2.3. Quantification of Nucleic Acid

2.3.1. DNA

DNA concentration was estimated by comparing the intensity of ethidium bromide fluorescent bands on 0.6% agarose gels made with TAE buffer when run against a known quantity of HindIII-digested λ DNA (NEB ladder).

2.3.2. RNA

RNA concentration was estimated spectrophotometrically where A_{260} (for a 1 cm path-length) of 1.0 corresponds to 40 µg/ml RNA. Concentrations were checked by comparing band intensity of ethidium bromide fluorescence on 1.4% agarose gels made with MOPS buffer when run against a known quantity of RNA ladder marker (NEB).

2.4. PCR

2.4.1. Genomic DNA

2.4.1.1. Reagents

Primers were purchased from MWG Biotech and instructions followed for use with the Expand High Fidelity PCR System (Roche Applied Science, UK). The QIAquick PCR Purification Kit (Qiagen) was employed to isolate the PCR product from the other contents of the reaction tubes.

2.4.1.2. Primers

All primers were purchased from NEB. Details of sequence position relative to gene and annealing temperatures used for PCR are given in table 2.1.

Table 2.1.

Primer	5'-3' Sequence	Purpose
1926F	GCGCGGTACCCCACGGTAGC ATCA	To demonstrate insertion of a 1.4kb Kan^R cassette within the coding region of *rpa*C in *Synechocystis* 6803 (that is, the mutant *Synechocystis* 6803 *rpa*C$^-$).
2005R	GCTAAAAGTCCGAGCTCCTT CTT	To demonstrate insertion of a 1.4kb Kan^R cassette within the coding region of *rpa*C in *Synechocystis* 6803 (that is, the mutant *Synechocystis* 6803 *rpa*C$^-$).
rpaCF (1) [-23→+7]	CATACACTGCAGAGGATTTT CATATGATGG	For generating a mutant of *Synechocystis* 6803 where *rpa*C may be expressed off the *psb*A2 promoter. Contains *Nde*I site at the start sequence of *rpa*C.
rpaCR (2)	CCTAAGATTCGGAGCTCATA TGCTAGCTAACAGAG	For generating a mutant of *Synechocystis* 6803 where

[+347→+313]		*rpa*C may be expressed off the *psb*A2 promoter. To amplify the coding region of *rpa*C. Contains stop sequence (antisense), *Hpa*I site for subsequent insertion of spectinomycin resistance cassette and *Sac*I site for cloning into pBluescript sk⁻.
psbA2F1 (5) 6970-6998 [-250→-231]	GTCGCCCCTCTGCAGAGCCC AGAACTATG	To amplify promoter region of *psb*A2 in *Synechocystis* 6803 *rpa*C⁻. Contains *Pst*I site for cloning product into pBluescript sk⁻.
psbA2R1 (6) 7260-7226 [+31→-4]	CGCTTTCGAGCTCTTGGAGA GTCGTTGTCATATGG	To amplify promoter region of *psb*A2 in *Synechocystis* 6803 *rpa*C⁻. Contains *Nde*I site for replacing *psb*A2 coding sequence with *rpa*C. Contains *Sac*I site for cloning product into pBluescript sk⁻.
psbA2F2 (7) 7215-7243 [-15→+15]	GGAATTATAACCATATGACA ACGACTCTC	To amplify coding region of *psb*A2 from *Synechocystis* 6803 *rpa*C⁻ in order to have aid insertion of the *rpa*C-spec^R construct by homologous recombination.

psbA2R2 (8) 7553-7574 +345→+324	GTGGAGCTCTACCAACTGGTAAGGACCACC	To amplify coding region of *psb*A2 from *Synechocystis* 6803 *rpa*C^{-} in order to have aid insertion of the *rpa*C-spec^{R} construct by homologous recombination. Contains *Sac*I site for cloning product into pBluescript sk^{-}.
1926internalF (3) 744926-744906 [+141→+162]	GTCTATGCCCTAGCCACAGTG	Sense strand of *rpa*C in *Synechocystis* 6803, to confirm by PCR correct size of *Synechocystis* 6803 *rpa*C^{-} $^{++}$ construct and extent of segregation.
1926internalR (4) 744906-744926 [+162→+142]	CACTGTGGCTAGGGCATAGAC	Antisense strand of *rpa*C in *Synechocystis* 6803, to confirm by PCR correct size of *Synechocystis* 6803 *rpa*C^{-} $^{++}$ construct and extent of segregation.
1846F 474288-474307 [-227→-208]	AGCGCAGCAACCATGGAACG	For generation of *Synechococcus* 7942 *rpa*C knock-out.
1846R 474964-474942	ATTGGTGCCTCGAGCATAGGTGC	For generation of *Synechococcus* 7942 *rpa*C knock-out. Contains *Xho*I

[+448→+427]		site.
1846AF	ACGCTCACATTTGGCGACAT	To amplify *rpa*C mRNA transcript in *Synechococcus* 7942.
1846AR	AGATTCTGCACTCGCTTCGA	To amplify *rpa*C mRNA transcript in *Synechococcus* 7942.
2792F (psbAIF)	GCTGTACAACGGTGGTCCTT	To amplify *psb*AI mRNA transcript in *Synechococcus* 7942.
2792R (psbAIR)	CCGATCGGGTAGATCAGAAA	To amplify *psb*AI mRNA transcript in *Synechococcus* 7942.
7942rrna16SF	ATGGCCAACTTCCATGGTGT	To amplify 16S rRNA mRNA transcript in *Synechococcus* 7942.
7942rrna16SR	TGGGCTACACACGTACTACA	To amplify 16S rRNA mRNA transcript in *Synechococcus* 7942.
aadAF (9)	CGCCGAAGTATCAACTCAAC	Sense strand primer within spectinomycin resistance cassette used in generating *Synechocystis* 6803 *rpa*C^{-++}.

aadAR (10)	GTTGAGTTGATACTTCGGCG	Antisense strand primer within spectinomycin resistance cassette used in generating *Synechocystis* 6803 *rpa*C^{-++}.
KanRev	CCTTCTTCACGAGGCAGACC TCAGCG	Primer within kanamycin resistance cassette, used for confirming *Synechococcus* 7942 *rpa*C^{-} genotype.
T3	AATTAACCCTCACTAAAGGG	Primer for sequencing plasmid constructs
T7	GTAATACGACTCACTATAGG GC	Primer for sequencing plasmid constructs

** Numbers in () refer to primer numbers in chapter 5, figure 5.1.

**Numbers below name refer to position in genome

**Numbers in [] refer to position relative to start of open reading frame.

2.4.1.3. Reaction conditions

The following protocol was used for Perkin Elmer thermocyclers:

94°C melting temperature for 5 minutes followed by the addition of 1 µl Taq polymerase.

2 minutes at 94°C.

25 cycles of: 30 s at 94°C, 30 s annealing temperature (varied with respect to primer, see table 2.1), 45 s at 72°C (unless otherwise stated).

7 minutes at 72°C.

2.4.2. Quantitative PCR

50 ng-2 μg total RNA was converted to cDNA using 4 units of reverse transcriptase (Omniscript, Qiagen) using 1 μM concentration of the appropriate primer. ≤ 1/10 of the reaction products was used in PCR using appropriate forward and reverse primers to determine transcript levels.

2.5. Restriction digests

Digests were carried out in 20 μl reaction volumes, using 1X recommended buffer in conjunction with 0.8 units of enzyme. 10X BSA buffer was added where appropriate. Restriction endonucleases were purchased from New England BioLabs (NEB). Digests were carried out at 37°C for 2 hours unless otherwise specified by NEB.

2.6. Ligations

Ligations were carried out in 20 μl reaction volumes at room temperature for 15 minutes. Reaction tubes contained approximately 100 ng of vector and approximately three times this quantity of insert. NEB Quick ligase (0.8 Units) was used in conjunction with the recommended buffer.

2.7. Transformations

2.7.1. *E.* coli

XL10-Gold Ultracompetent cells were purchased from Stratagene. Ligation mixtures were added to the fresh competent cells, incubated on ice for 30 minutes. The cells were exposed to a 30 s heat shock at 41°C, and given 2 minutes to recover on ice, before 0.9 ml of LB medium (pre-warmed to 37°C) was added. The cells were incubated in a shaking incubator at 37°C for 1 hour, and plated onto selective LB agar plates, containing the 100μg/ml ampicillin, and 50 μg/ml of kanamycin and/or specinomycin, where appropriate.

2.7.2. Cyanobacteria

Cells from cultures in exponential growth phase were concentrated to an OD750 of 1.0 and 1 μg DNA added to 1 ml of cells in moderate light. Following a 4 hour

incubation at 30°C in a stationary incubator (inverting the tube occasionally), cells were spread plated onto non-selective agar. After 72 hours, 2 ml of top-agar containing the appropriate concentration of antibiotic was laid onto the plates. Transformants were picked as single colonies, and plated to fresh selective agar plates at least twice before liquid cultures were started.

2.8. DNA Sequencing

Plasmid DNA was extracted via the protocol in 2.2.1.2. 200 fmoles double-stranded DNA was sequenced using or T3 or T7 primers (2-5 pmol/μl) by the Wolfson Institute for Biomedical Research, University College London. The sequence was analysed using Chromas 1.45 (Conor McCarthy, Griffith University, Queensland, Australia).

2.9. Quantification of Pigment

2.9.1. Chlorophyll-*a* Concentration

This was obtained using the method of Porra *et al* (1989), where 1 ml of cell culture was harvested using a microfuge (Heraeus Instruments, UK), re-suspended in 1 ml of methanol before being thoroughly vortexed and centrifuged again. The absorbance at 665 nm of the resulting supernatant multiplied by 14.0 corresponded to the concentration (μM) of chlorophyll *a*.

2.9.2. Phycocyanin/Chlorophyll *a*

This was calculated using absorption spectra of intact cells, and the formula described by Myers *et al* (1980).

2.9.3. PSI

PSI was quantified spectrophotometrically using a machine designed by Professor Peter Rich, University College London. A known quantity of cells, suspended to a total chlorophyll concentration of 10 μM was incubated for 15 s in the dark in the

presence of 50 μM DCMU. The cells received eight saturating 6 μs flashes of blue light generated using a BG39 filter over white light from a xenon flash lamp in order to completely oxidise P700 of PSI. The red wavelength detection was generated using a 695 nm cut-on filter and a narrow band 695-703 nm notch filter. The amplifier constant was $1x10^5$ with a 10 μs time constant. A 2 s sweep (on the transient recorder) of 8 cycles was averaged to one trace. An absorbance difference between values before and after flashing, multiplied by the extinction coefficient of 64 $mM^{-1}cm^{-1}$ (Hiyama and Ke, 1972) allowed an accurate estimation of PSI content to be determined.

2.9.4. PSII

This was facilitated according to the binding properties of ^{14}C-labelled atrazine, and based upon the method employed by Chow *et al* (1990). A known quantity of cells was harvested to 20μM chlorophyll *a*. 1 ml aliquots were dark-adapted and incubated in the presence of varying amounts of radio-labelled atrazine (50-400 pmol). The supernatants from a centrifugation step, containing atrazine which failed to bind to the Q_B site of PSII were mixed with a scintillation cocktail purchased from Sigma-fluor and counted. Saturating quantities of bound atrazine can be estimated as being equivalent to the PSII content of the cells. A reference assay of known dilutions of atrazine was used, and saturation was estimated by a weighted linear regression on a double-reciprocal plot of atrazine added : atrazine bound. Atrazine may bind non-specifically to other quinone-binding sites as the amount of atrazine is increased. However, since it binds far more specifically to the Q_B site of PSII, and that the amount of bound atrazine is estimated from the part of the plot before saturation phase, this error is minimised.

2.10. Determination of Oxygen Evolution

1ml Cyanobacterial samples of 20μM Chlorophyll *a* were tested using the OXYLAB2 liquid-phase Oxygen electrode (Hansatech, Norfolk, UK). Light intensity and temperature were monitored using a Quantitherm meter (Hansatech, Norfolk, UK). For oxygen evolution, rate measurements were recorded at increasing light intensities, to saturating levels at 30°C. Units for Oxygen evolution

are expressed throughout in μmoles oxygen/ml/minute. To convert to μmoles oxygen/mg chlorophyll/hour, data may be multiplied by 2.978 x 10^{-4}.

2.11. Fluorescence Spectra

These were obtained in a Perkin-Elmer LS50 Luminescence Spectrometer (Foster City, CA) possessing a modulated light source with a lock-in amplifier.

2.11.1. Room temperature time-course (25°C)

Cells were suspended to a known chlorophyll concentration (5 μM unless otherwise stated) in a 3 ml fluorescence cuvette. Fluorescence measurements were obtained for wavelength values between 600 and 680 nm for 600 s using excitation light at 600 nm to excite the phycobilisomes. Data was recorded at intervals of 1s for this period, and adaptation to light-state 1 or light-state 2 (see 2.11.1 for details) was carried out at specified times. Slit widths were set at 10 nm and 10 nm for excitation and emission respectively.

2.11.2. Room temperature fluorescence emission spectra (25°C)

Cells suspended to 2.5 μM chlorophyll were used. For emission spectra, an excitation wavelength of 600 nm was used to excite phycocyanin. For chlorophyll *a*, an excitation wavelength of 435 nm was used. In both cases, emission was recorded between 620-750 nm. Excitation and emission slit-widths were set to 3nm and 5nm respectively.

2.11.3. Chlorophyll *a* fluorescence measurements (30°C)

Cells were maintained at 30°C, suspended to 2.5 μM chlorophyll and dark adapted for 20 minutes. Fluorescence was detected by PAM fluorimetry using a Hansatech FMS-1 fluorimeter (Hansatech, Norfolk, UK). For the purposes of monitoring non-photochemical quenching of F_0 and F_v, actinic white light (1000 $\mu mol.m^{-2}.s^{-1}$) was provided using a fibre optic suspended above the cells. Saturating pulses could be provided at 3000 $\mu mol.m^{-2}.s^{-1}$ for 1 second duration.

2.11.4. 77K emission spectra

Samples were grown to a total chlorophyll concentration of 5 μM. Cell suspensions were injected into silica capillary tubes of 2.5 mm internal diameter and frozen in liquid nitrogen.

For emission spectra, an excitation wavelength of 600 nm was used to excite phycocyanin or 435 nm for the excitation of chlorophyll *a*; emission was recorded over a wavelength of 620-750 nm. Both excitation and emission slit-widths were set to 5nm.

2.12. State transitions

2.12.1. Acclimation to light-state

Cyanobacterial cells grown in white light of 9 $\mu mol.m^{-2}.s^{-1}$ adapted to state 1 by exposure to light of intensity 20 $\mu mol.m^{-2}.s^{-1}$ from a Highlight 2000 lamp fitted with a long pass Schott RG665 red glass filter employed to permit transmission of light above 665 nm, so that cells were exposed to red light. Cells were routinely given 5 minutes to adapt to either state 1 (in red light) or state 2 (dark).

2.12.2. Arresting cells in light-state 1 or light-state 2

A suspension of cells corresponding to a concentration of 50 μM chlorophyll was light-adapted (as described in 2.11.1.) or dark-acclimated. A range of different osmotica were assayed to determine fixation. These were:

1. SPCM (0.5 M sucrose, 0.5 M Potassium di-Hydrogen phosphate, 0.3 M tri-Sodium citrate, 10mM Magnesium chloride, pH 6.8) (Gantt, 1988)

2. KH_2PO_4/K_2HPO_4 (pH 6.8, concentrations between 0.1 M and 0.5 M)

3. Sucrose (0.1 M – 1.0 M)

4. KCl (0.1 M – 1.0 M)

The solution was added following the initial acclimation step, to give a final chlorophyll concentration of 5 μM. The cell suspension was maintained under the same illumination conditions for a further 5 minutes.

2.12.3. Kinetics of the transition to state 1

A laboratory-built fluorimeter (Peter Rich, University College London) permitted fluorescence transients to be recorded at room temperature. Cells were suspended to a chlorophyll concentration of 30 μM in BG11, then dark adapted for 5 minutes. Nine volumes of phosphate buffer were then added whilst maintaining the suspension in the dark a further 5 minutes. DCMU was added to a final concentration of 50 μM. Illumination was provided and phycobilin-absorbed light selected using a combination of Schott RG610 and Ealing 660 nm short pass filters. The illumination was controlled using a shutter that was operated electronically. This shutter opened in approximately 1ms and allowed the cells to be provided with light at an intensity of 100 $\mu mol.m^{-2}.s^{-1}$. The PSII fluorescence was selected using a Schott RG695 red glass filter and detected by a photomultiplier.

2.13. Fluorescence Recovery After Photobleaching (FRAP)

2.13.1. Preparation of samples

Cells grown and adapted under the desired illumination conditions were adsorbed onto 1.5% agar containing the appropriate media. Where appropriate, cells were grown in the presence of 0.5% DMSO, inhibiting cell division and therefore elongating the cells (Sarcina and Mullineaux, 2000). A small block of the agar was cut from the Petri dish and placed in a sample holder (S. Garcia, University College London). A 0.2mm thick coverslip was placed on top of the agar block, and the measurements were obtained with the sample holder on a microscope stage heated to 30°C (for *Synechococcus* 7942 and *Synechocystis* 6803) using a circulating water-bath. In order to permit observation at high magnification, a drop of immersion oil was placed between the coverslip and the 60X objective lens.

2.13.2. FRAP measurement: phycobilisomes

A red He-Ne laser (633 nm, 20 mW) was used in conjunction with a scanning confocal microscope (Nikon PCM2000). An RG650 dichroic was used in addition to an RG665 red emission filter to select wavelengths above 665nm. Focused laser light was directed through a pinhole of 50μm, and finally towards the sample.

A sample aligned parallel to the y-axis was bleached by scanning the laser through the middle of the cell in a single dimension (x-plane) for 2 seconds. A neutral

density filter facilitated the intensity of laser light being reduced (to 12.5% transmission) to prevent further photobleaching. For the sample region (20.5 x 20.5 μm), images (512 x 512 pixels) were obtained in the x-y plane using the scanning microscope every 3 seconds for 30 seconds post-bleach, and then at 5 minute intervals for a duration of 30 minutes where appropriate.

Each image was saved as a colour "ids" file initially, then saved again as an 8-bit grey scale "tiff" file. Cells not aligned precisely in the y-direction were rotated appropriately and re-saved as 8-bit grey scale tiff files on a black background. The tiff files were opened using Optimas 5.2 imaging software (Optimas Corporation).

Optimas 5.2 imaging software was used to determine the one-dimensional bleaching profile, and Sigmaplot version 5.0 (Jandel Scientific, San Rafael, California, USA) fitted this profile to a Gaussian curve in order to determine the width and depth of the bleached region and finally to enable determination of the diffusion coefficient. Box 2.1. details how raw fluorescence data may be processed to achieve this (Mullineaux *et al*, 1997; Mullineaux, 2004).

2.13.3. FRAP Measurement: chlorophyll *a* (PSII)

As in 2.11.2, but a 100mW Argon laser was used (457 nm) in conjunction with a 475 nm dichroic and an RG665 red emission filter, transmitting wavelengths above 665 nm.

Box 2.1. (See Figure 2.1 for graphical representation)

To determine diffusion rates of chromophores, fluorescence profiles must be determined. A fluorescence profile is generated by drawing a vertical line thick enough and long enough to encompass the entire cell, the Optimas software extracting the pixel intensity information. Figure "a" shows a pre-bleach (grey) and post-bleach (black) fluorescence profile at time t = 0 s. (Note: This time point is dubbed t = 0 s, but some information may be lost in the time between cessation of bleaching and recording of the first image if the chromophores are indeed mobile.) Figure "b" features the images of the cell at these two stages. Scale bar = 4 μm.

If chromophores are mobile, the dip in the post-bleach fluorescence profile will become broader and shallower, as shown in figure "c" Here, the red trace corresponds to fluorescence along the cell at time t = 12 s. If overall fluorescence along the cell has faded over time, the fade factor estimated by deducing fluorescence of a cell exposed to the laser but not bleaching is determined and the fluorescence of the experimental cell adjusted accordingly.

The difference between the initial fluorescence and each fluorescence post-bleach profile may be determined, where the greatest difference will be between pre-bleach and time t = 0 s. The data are fitted to Gaussian curves at this point, and estimates of the depth of the bleach, width and midpoint of the bleach can be made. Figure "d" depicts this. The raw data difference in fluorescence is plotted in black, and the fitted line in grey.

Regression analysis may be achieved, fitting these data and the time points when they were recorded to a straight line, denoted by figure "e".

D, the diffusion coefficient can be calculated using the following equation:

$C_t = C_0R_0(R_0^2 + 8Dt)^{-½}$,

where C_t = bleach depth at time t seconds, C_0 = initial bleach depth, R_0 = initial half-width of the bleach (Mullineaux *et al*, 1997).

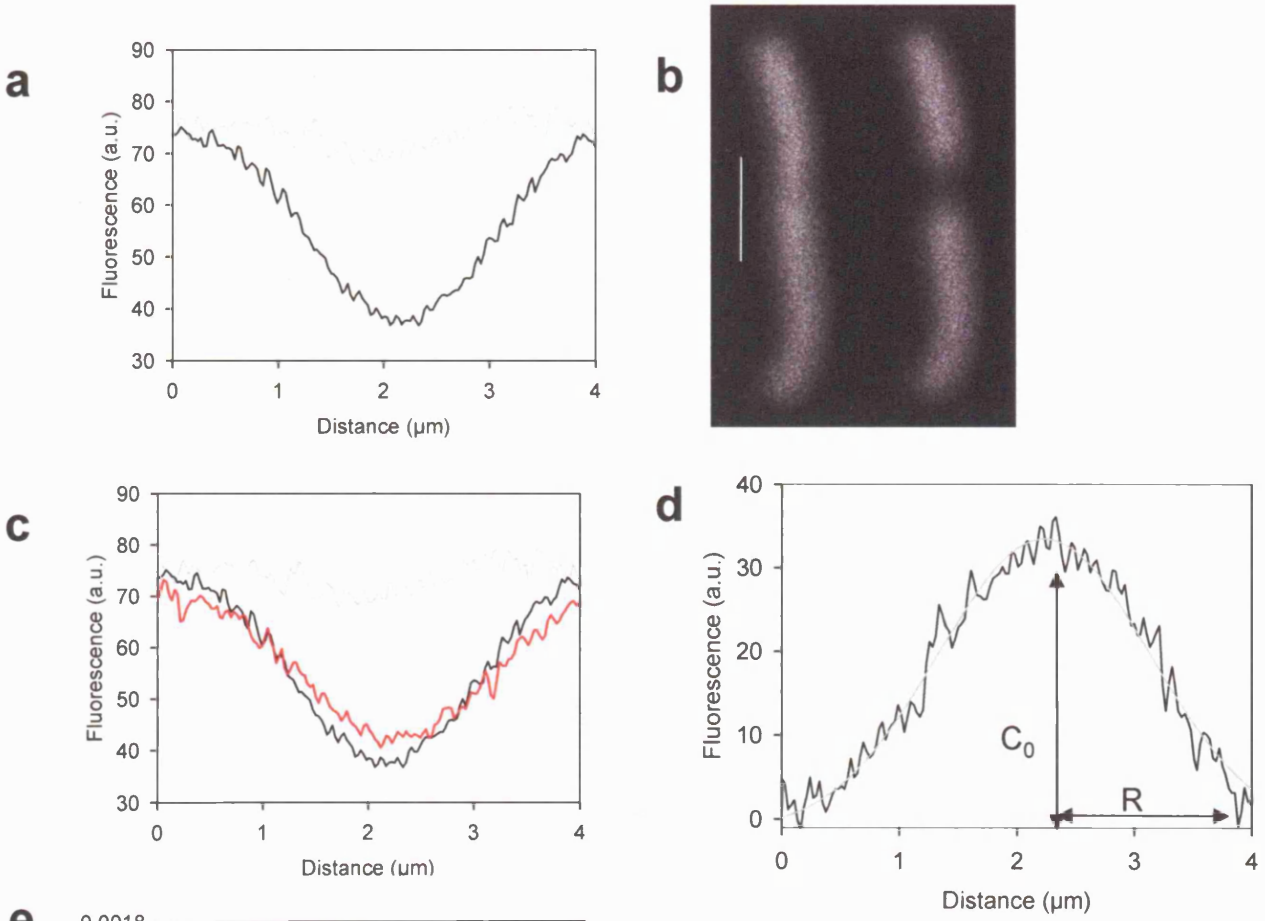


Figure 2.1. Summary of FRAP. (See box 2.1 for details)

2.14. Extraction of cyanobacterial thylakoid membranes with functionally coupled phycobilisomes

50 ml of dense cell culture was harvested by centrifugation and (light/dark-adapted if appropriate) re-suspended in 10 ml SPCM (Gantt, 1988), centrifuged again and concentrated two-fold. The suspension was treated twice at 13.5 kpsi in a Constant Cell Disrupter (Warwickshire, UK). The thylakoid membranes were obtained in two centrifugation steps. The first step was conducted at 30000 rpm with a Sorvall-SS34 rotor for 15 minutes at 4^0C in a Sorval Evolution RC Centrifuge (Hitachi, Hertfordshire, UK). Ultracentrifugation of the supernatant using a Sorvall-T657 for 45 minutes at the same temperature in a Sorvall Discovery SE (Hitachi, Hertfordshire, UK) followed this. The second step served to pellet the membranes which were re-suspended in 50 µl SPCM.

2.15. Extraction of phycobilisome-free thylakoid membranes

50 ml of dense cell culture was harvested by centrifugation and re-suspended in 50 ml buffer containing 25% glycerol, 10 mM $MgCl_2$, 50 mM HEPES, pH 7.5. The cells were broken using the cell disrupter twice at 13.5 kpsi, and centrifuged at 30000rpm with a Sorvall-SS34 rotor for 15 minutes at 4°C. The supernatant was centrifuged with a T-865 rotor at 4°C, 40000 rpm for 45 minutes. The pellet from this ultracentrifugation step was re-suspended in the same buffer, homogenised and centrifuged again, the resultant pellet being re-suspended in 10 mM Tris (pH 6.8).

2.16. Imaging of thylakoid membranes using atomic force microscopy

Cyanobacterial membranes were extracted from whole cells using the methods described in section 2.14 or 2.15, and suspended to a chlorophyll concentration of 5 mg/ml. Membrane suspensions were diluted 100-fold in 10 mM Tris (pH 6.8) with varying concentrations of KCl (50 mM to 500 mM) and 20 µl of the diluted stock was adsorbed to freshly cleaved mica mounted onto a circular metal disc with superglue. Membranes were given 40 minutes to adsorb before being placed in an atomic force microscope. Cantilevers with sharpened SiN_4 tips were used, and the tip was given 1 hour to equilibrate in buffer with the membrane sample. A set force of 0.1 V was applied, and the sample scanned at a rate of 1Hz. Images generated of 512 x 512 pixels, and the resolution calculated according to the actual area of the

sample scanned. The true height of a sample of membranes equates to the measured heights in high salt concentrations.

Chapter 3: Phycobilisome Mobility and State Transitions

Chapter 3: Phycobilisome Mobility and State Transitions

3.1. Objective

- To establish whether phycobilisome mobility is required for state transitions

3.2. Introduction

State transitions have been observed in both eukaryotic and prokaryotic photosynthetic organisms. In the case of *Chlamydomonas*, a eukaryote, there is evidence that the light harvesting complex, LHCII, moves between the reaction centre complexes, PSI and PSII to trigger a state transition (Keren and Ohad, 1998).

The issue of the cyanobacterial state transition has until now been far more controversial. One of the main mechanistic hypotheses is the spill-over model, where the light harvesting complexes, the phycobilisomes, are fixed onto PSII, and that during a transition to state 2, a little excitation energy spills over to PSI (Bruce *et al*, 1989, for example). The other hypothesis relies on phycobilisomes being mobile, transiently associating with one or the other reaction centre according to different wavelengths of illumination (Tsinoremas *et al*, 1989; Zhao *et al*, 1991; Mullineaux, 1994, for example). Support for this model of the state transition is given by evidence in *Dactylococcopsis salina* and *Synechococcus* sp. PCC 7942 that phycobilisomes are able to diffuse on the thylakoid membranes (Mullineaux *et al*, 1997; Sarcina *et al*, 2001; Aspinwall *et al*, 2004, for example), coupled with the fact that PSII does not diffuse under the same conditions (Mullineaux *et al*, 1997; Sarcina *et al*, 2001). There is also a great deal of evidence that phycobilisomes transfer energy directly to both photosystems without a decrease in energy storage (Mullineaux *et al*, 1994).

Also contentious is how the phycobilisomes couple to a reaction centre and then de-couple, should a mobile model be applicable to state transitions in cyanobacteria. In eukaryotes, the reversible phosphorylation of LHCII determines to which reaction centre excitation energy will be transferred. It is unknown whether phosphorylation is involved in cyanobacteria. It is also uncertain as to whether phycobilisomes or reaction centres, particularly PSII, undergo a conformational change in order to increase or decrease the strength of interactions. It has been

suggested that the strength of interaction between phycobilisomes and PSII is more critical than to PSI, since a PSII$^-$ mutant of *Synechocystis* sp. PCC 6803 behaves like the wild type in experiments designed to examine energy transfer to PSI (Mullineaux *et al*, 1994). Evidence presented in chapter 4 also suggests that affinity of the phycobilisomes for PSII is greater than for PSI.

3.3. Rationale

Previous studies (Mullineaux, 1993; Li *et al*, 2004) have shown that buffers of high osmotic strength

a) inhibit state transitions
b) arrest cells in a light-state 1 or state 2 depending on illumination prior to buffer exposure

Given that large scale phycobilisome mobility is observable using FRAP, it is possible to determine whether such buffers inhibit the movement of the light harvesting apparatus over the same concentration range, and thus, whether diffusion of phycobilisomes is critical to the observation of state transitions. The possibility of different conformations of phycobilisomes in the different light states will also be examined.

3.4. Materials and Methods

See sections 2.10.2., 2.11.1., 2.11.3., 2.12.1. and 2.12.2. for details.

3.5. Results

3.5.1. Fixation of the state transition using high osmotic strength buffers

It is possible to record state transitions by observing differences in fluorescence peaks of 77K fluorescence emission spectra (Murata, 1969). In state 1, there is a greater observable fluorescence of PSII than there is when cells are adapted to state 2. Treatment of state 1 or 2 adapted *Synechococcus* sp 6301 cells with phosphate buffer was previously shown to inhibit re-adaptation to the alternate state in a concentration dependent manner (Mullineaux, 1993). The data presented here confirms this observation for *Synechococcus* sp PCC 7942 and *Synechocystis* PCC 6803, showing that the effect is not species specific.

Synechococcus 7942 cells were adapted to state 1 by exposure to red light for five minutes or state 2 in the dark (for the same duration) and then treated with a phosphate buffer (KH_2PO_4/K_2HPO_4), pH 6.8. Buffer concentrations tested were: 0.1 M, 0.2 M, 0.3 M, 0.4 M and 0.5M. After 5 minutes in the buffer under the same illumination conditions, treated cells were then either maintained in the same conditions, or re-adapted under the alternate illumination conditions prior to being frozen in liquid nitrogen. In state 1, the fluorescence emission spectra obtained at 77K show a larger proportion of fluorescence at 680-695 nm (from PSII) compared with that observed at 720 nm (PSI) than when adapted to state 2 (figure 3.1).

State transition fixation was estimated according to the ratio of fluorescence peaks obtained at 685nm from the 77K fluorescence emission spectra. Since FRAP data were to be obtained (figure 3.8-3.10) on cells grown in the presence of 0.5% DMSO, for control purposes, fluorescence emission spectra were obtained for cells in grown in the presence or absence of the cell division inhibitor. Figure 3.2 demonstrates that DMSO does not inhibit cells from carrying out state transitions. Figure 3.3B showed that the spectrum for cells adapted to state 1 remains the same following the addition of 0.5 M phosphate regardless of whether they were maintained in red light (state 1 conditions) or in the dark (state 2) subsequent to treatment. This is not the case where cells have been provided with normal growth medium instead (figure 3.3A).

It is, however, important to note that the prominence of the peak at 685 nm increased when cells had been treated with 0.5 M phosphate. This effect was not observed when chlorophyll was excited at 435 nm. When exciting the phycobilisomes at 600 nm, part of the fluorescence at 685 nm comes from the terminal emitters from the core of the phycobilisome. Given that this peak was increased relative to the peaks at 695 nm and 710 nm (corresponding to PSII and PS1 respectively), it suggests that energy transfer from the phycobilisomes to the reaction centres was less efficient when cells were phosphate-treated.

It is possible to determine the minimum concentration of phosphate required for arresting cells in state 1 or state 2 by estimating the degree to which cells are fixed in the light state from the 77K emission spectra. The emission spectra were

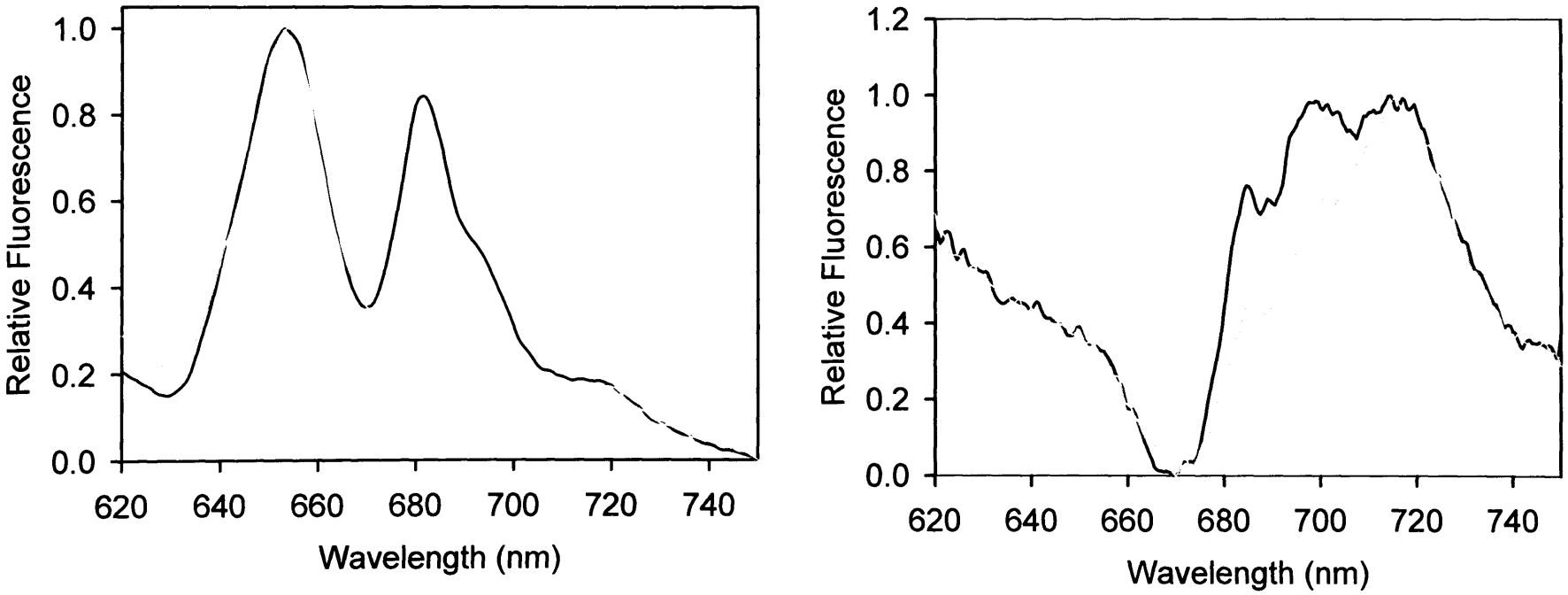


Figure 3.1. 77K whole cell fluorescence emission spectra for *Synechococcus* 7942 excitation at a) 600nm (phycobilisomes) and b) 435nm (chlorophyll) showing cells in state 1 (black) and state 2 (grey). Spectra were obtained using excitation and emission slit widths of 5nm, and in a) were normalised to phycocyanin fluorescence at 650nm, and b) to the PSI peak at 720nm.

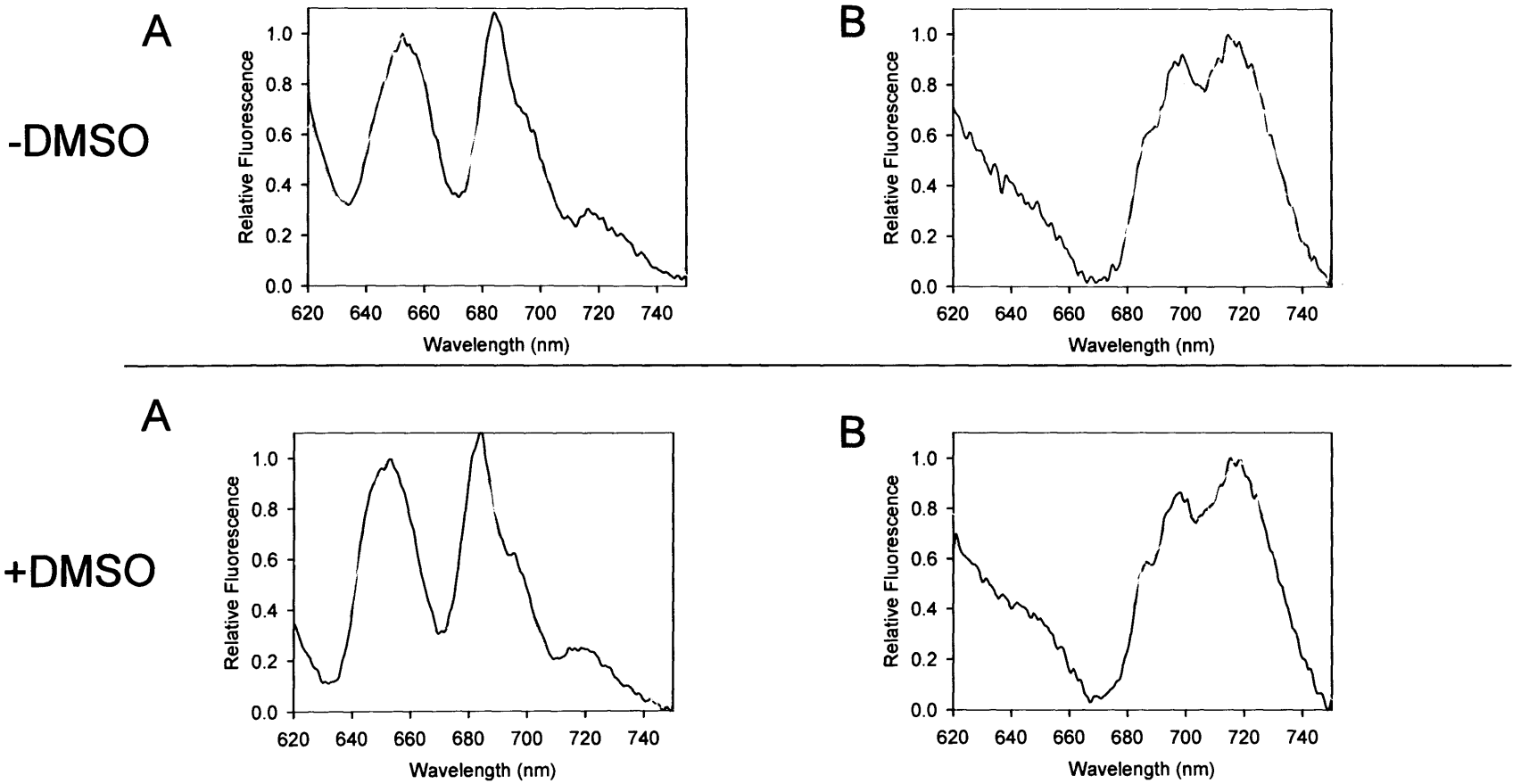


Figure 3.2. 77K whole cell fluorescence emission spectra for *Synechococcus* 7942 excitation at a) 600nm (phycobilisomes) and b) 435nm (chlorophyll) showing cells in state 1 (black) and state 2 (grey). Spectra were obtained using excitation and emission slit widths of 5nm, and in a) were normalised to phycocyanin fluorescence at 650nm, and b) to the PSI peak at 720nm. Cells were grown under low intensity white light with (+) or without (-) 0.5% DMSO.

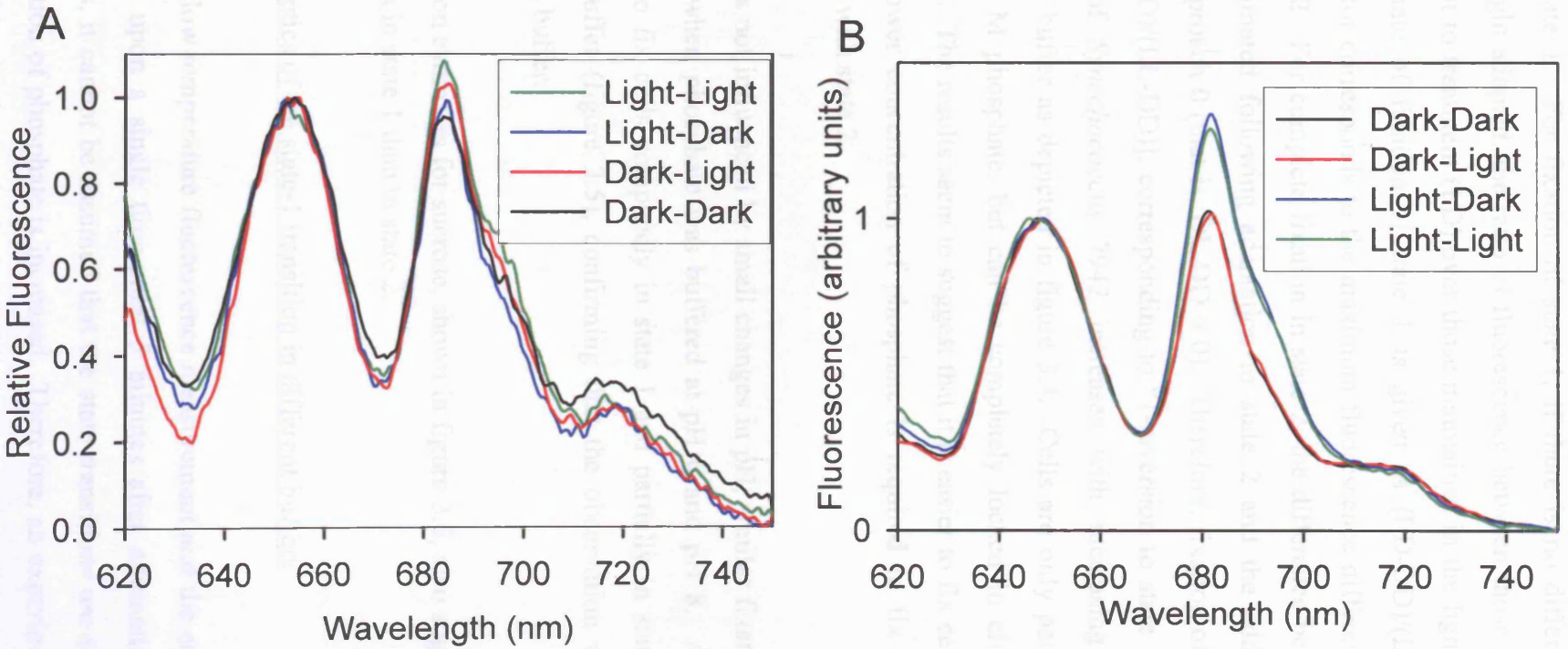


Figure 3.3. 77K whole cell fluorescence emission spectra for excitation at 600nm of *Synechococcus* 7942 cells acclimated to either light (state 1) or dark (state 2) before being treated with BG11 (A) or 0.5M phosphate (B). The same conditions were then either maintained or changed following 5 minutes in the phosphate buffer.

normalised to the phycocyanin fluorescence peak at 654 nm. A higher ratio of fluorescence obtained for emission at 685 nm to 654 nm is indicative of adaptation to light-state 1. For fixation of state 1, if there is no difference between cells initially light adapted, the ratio of fluorescence between those adapted in the dark subsequent to treatment (LD) over those maintained in the light (LL) should be 1. The estimate of fixation of state 1 is given as (LD-DD)/(LL-DD), where the denominator corresponds to the maximum fluorescence difference between state 1 and state 2. For complete fixation in state 2, the difference between cells light or dark acclimated following adaptation to state 2 and the addition of phosphate should approach 0 (that is, DL-DD = 0). Therefore, fixation of state 2 is given by 1-[(DL-DD)/(LL-DD)], corresponding to "1-reversion to state 1". State transition fixation of *Synechococcus* 7942 increases with increasing concentrations of phosphate buffer as depicted in figure 3.4. Cells are only partially fixed in state using 0.1 M phosphate, but can be completely locked in either state at 0.4 M phosphate. The results seem to suggest that it is easier to fix cells in state 1 than 2, since a lower concentration of phosphate is required to fix cells in state 1 as compared with state 2.

Fixation is not influenced by small changes in pH; similar fixation properties were recorded when phosphate was buffered at pH 6 and pH 8. Additionally, it was possible to fix cells completely in state 1 and partially in state 2 using a 0.5 M sucrose buffer (figure 3.5), confirming that the observation was not specific to phosphate buffer.

The fixation estimates for sucrose, shown in figure 3.6, also suggest that it is easier to fix cells in state 1 than in state 2.

3.5.2. Kinetics of the state-1 transition in different buffers

Since the low temperature fluorescence measurement and the estimates for fixation are based upon a single time-point 5 minutes after alternating the illumination conditions, it cannot be assumed that the state transitions are slowing down as the concentration of phosphate is increased. Therefore, an experiment to monitor state transition kinetics is required. A reliable way to achieve this is to monitor the

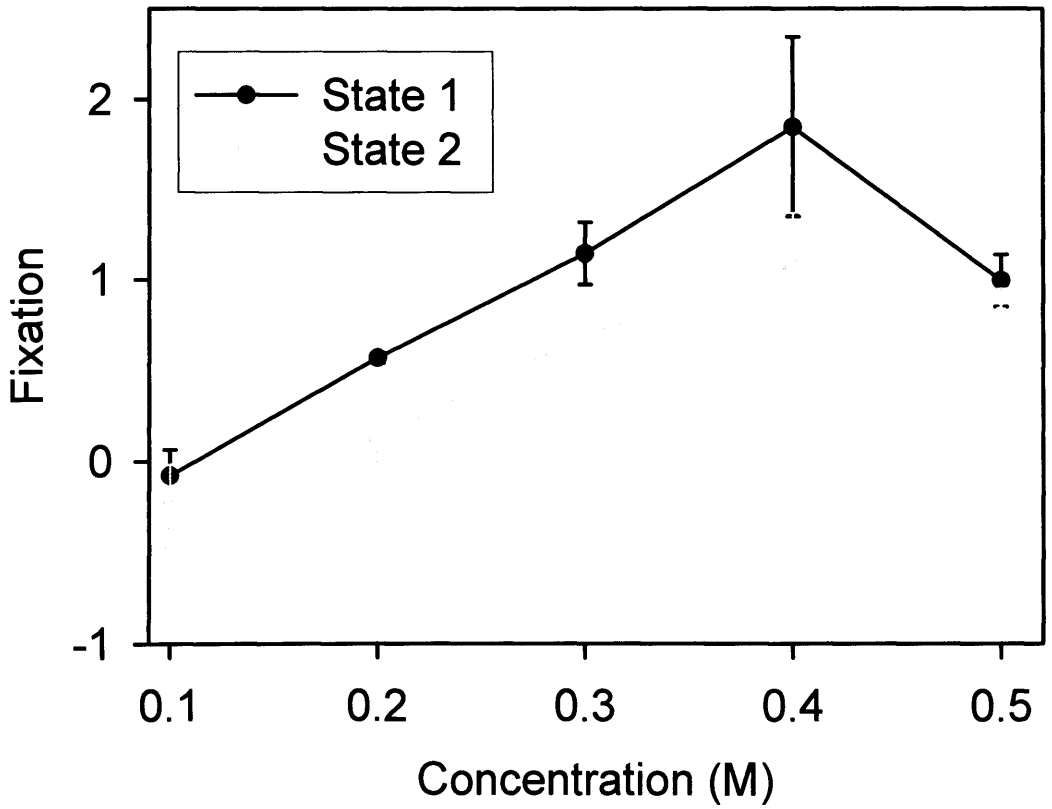


Figure 3.4. Fixation of the light-state using phosphate. n=6 for each concentration, with standard deviations given

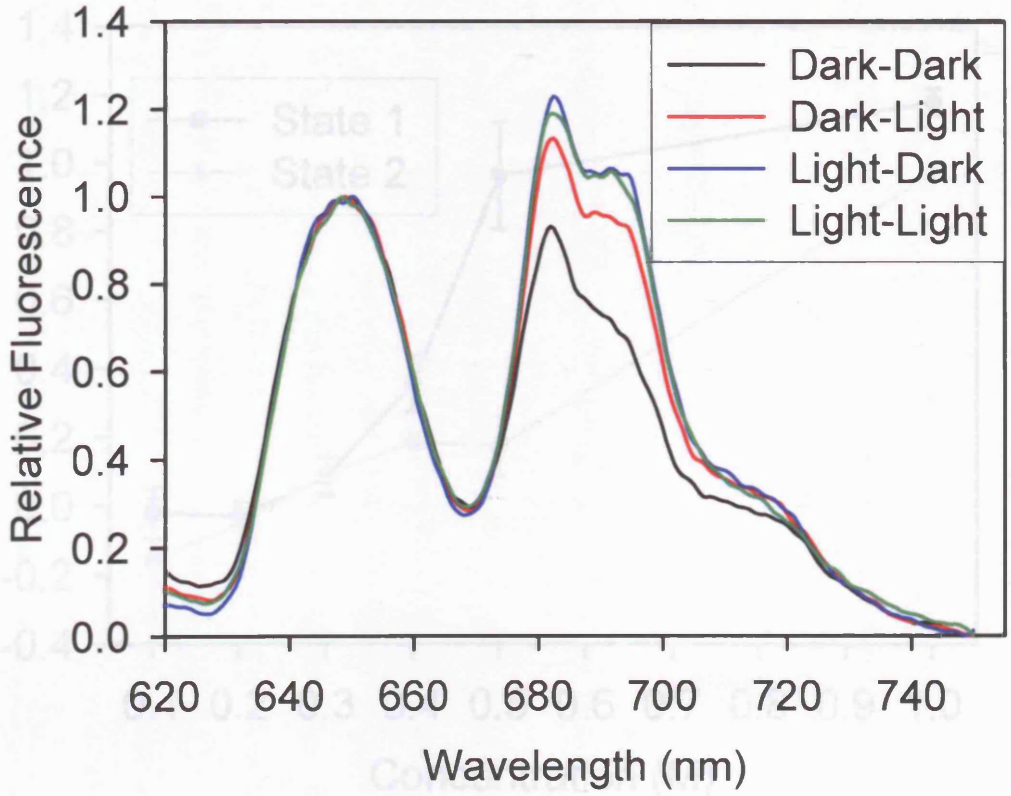


Figure 3.5. 77K whole cell fluorescence emission spectra for excitation at 600nm of *Synechococcus* 7942 cells treated with 0.5M sucrose.

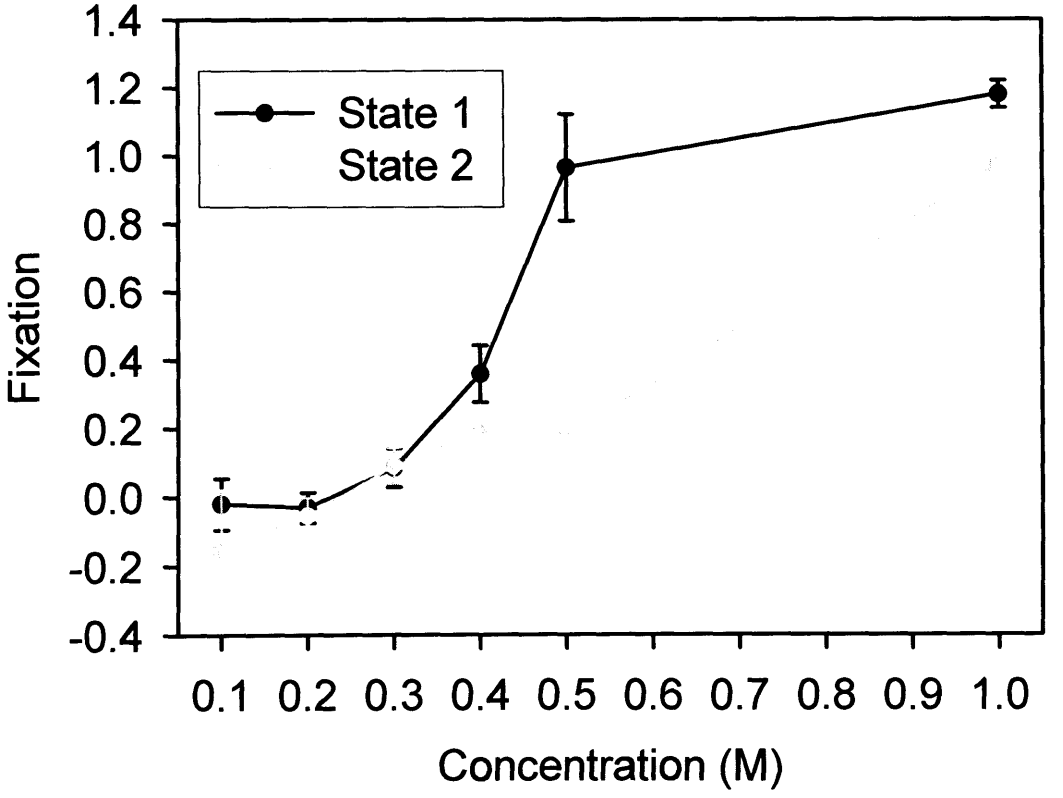


Figure 3.6. Fixation of the light-state using sucrose. n=6 for each concentration, with standard deviations given

fluorescence increase observed when the transition from state 2 to state 1 is made over a timescale of seconds to minutes at room temperature. Phosphate treated cells in state 2 were exposed to DCMU in order that upon red light illumination, electron transport out of PSII would be blocked, and the gradual rise in fluorescence which follows and corresponds to the state transition could be monitored (Schluchter *et al*, 1996; Aspinwall *et al*, 2004). The fluorescence transients obtained feature in figure 3.7., and show that the state transition does indeed slow between 0.1 M phosphate and 0.3 M phosphate, the latter result indicating that a state transition is not occurring.

Higher concentrations of sucrose and KCl are required in order to stop cells from making the transition to state 1. In both cases, state transitions are no longer observed between 0.7 M and 0.8 M concentrations of these solutions.

3.5.3. Phycobilisome mobility

3.5.3.1. Phycobilisome mobility and buffers of high osmotic strength

It has been shown using a technique called FRAP that phycobilisomes are able to diffuse (Mullineaux *et al*, 1997). This technique relies upon a laser bleaching out the fluorescent pigments in a line uni-directionally through an elongated cell. The rate of fluorescence recovery in the bleached region can be determined imaging phycocyanin fluorescence using a laser scanning confocal microscope fitted with a 633 nm red HeNe laser which is capable of exciting the phycocyanin pigments. Using the images obtained, the fluorescence differences (pre-bleach and at known times post-bleach) were fitted to Gaussian curves. In order to establish whether phycobilisome mobility is required for state transitions, *Synechococcus* sp 7942 cells were treated with varying concentrations of phosphate and a mean phycobilisome diffusion coefficient was obtained from 6 FRAP measurements. The cells had been elongated by growth in BG11 supplemented with 0.5% dimethylsulfoxide; this chemical does not cause any noticeable alterations to the structure of the thylakoid membranes or photosynthetic capability as shown by fluorescence imaging, electron microscopy and oxygen evolution compared with untreated cells (Mullineaux and Sarcina, 2002). It is therefore convenient to use for

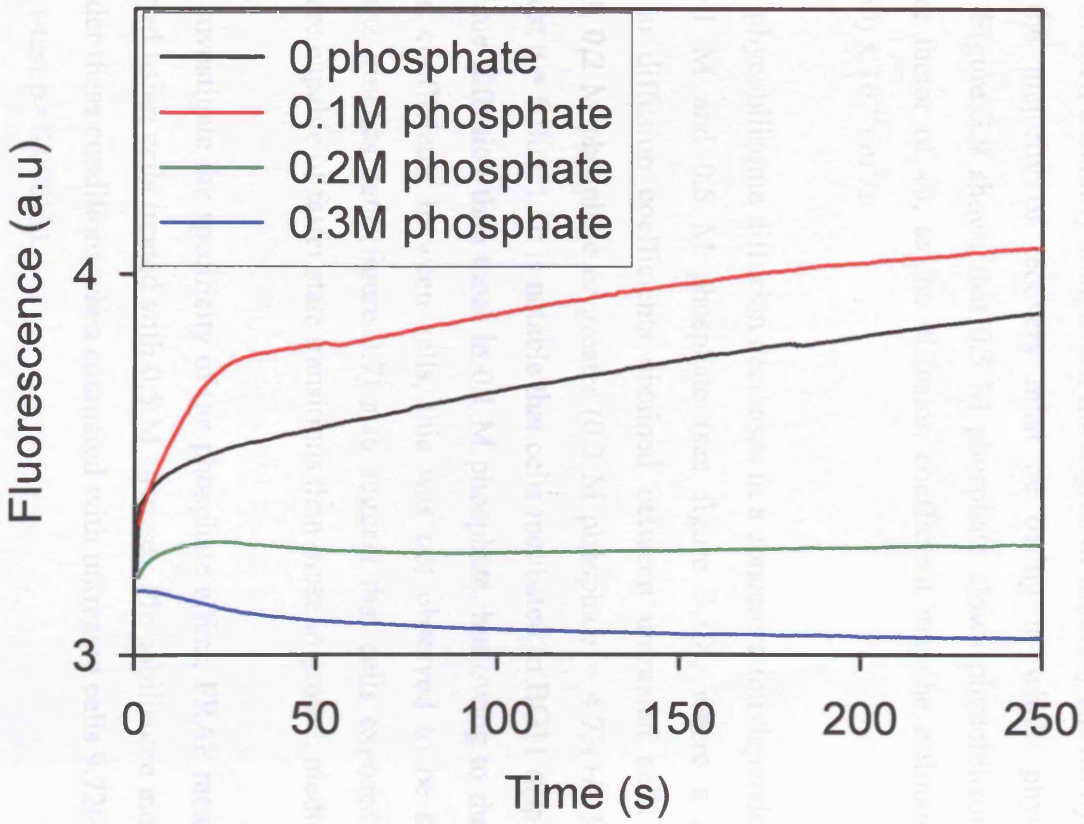


Figure 3.7. Kinetics of the transition to state-1 for dark adapted phosphate-treated cells. DCMU was provided prior to illumination at time 0.

FRAP measurements, where longer cells give better approximations to theoretical distributions.

The diffusion coefficient of phycobilisome movement under normal conditions (figure 3.8) was estimated in this study to a mean of 4.32(+/-1.72) x $10^{-10}cm^2/s$, a figure comparable with measurements obtained previously (Sarcina *et al*, 2001; Aspinwall *et al*, 2004). It should be noted that while a small amount of the observed fluorescence recovery by phycobilins may be attributable to the movement of their assembly complexes, the degree of mobility shown by cells is so extensive the majority of recovery must be owing to whole phycobilisome diffusion. Figure 3.9 shows that 0.5 M phosphate slows phycobilisomes by an approximate factor of 40, as the diffusion coefficient may be estimated here at 9.80(+/-1.60) x $10^{-12}cm^2/s$.

The rate of phycobilisome diffusion decreases in a concentration dependent manner between 0.1 M and 0.5 M phosphate (see figure 3.10), where a significant difference in diffusion coefficients obtained between untreated cells and cells treated with 0.2 M phosphate or greater (0.2 M phosphate = 4.73(+/- 3.23) x $10^{-11}cm^2/s$; t-test p = 0.0003). It is notable that cells incubated in BG11 display slower phycobilisome diffusion than those in 0.1 M phosphate, but owing to the variation in diffusion coefficients between cells, this was not observed to be significant. However, the kinetics data (figure 3.7) also suggest that cells exposed to 0.1 M phosphate are capable of faster state transitions than those in growth medium.

In order to investigate the specificity of the phosphate effect, FRAP measurements were obtained using cells treated with 0.5 M sucrose. Phycobilisome mobility was reduced under these conditions when compared with untreated cells 9.72(+/- 0.64) x $10^{-12}cm^2/s$; t-test p= 0.00013).

The inhibitory effect of the buffer on phycobilisome mobility is reversible; phosphate treated cells undergoing a FRAP measurement on BG11 agar exhibit diffusion coefficients comparable with untreated cells on the same growth medium (treated = 6.02(+/- 3.32) x $10^{-10}cm^2/s$; t-test P = 0.3), owing to the phosphate having been sufficiently diluted out to negate the inhibitory effect.

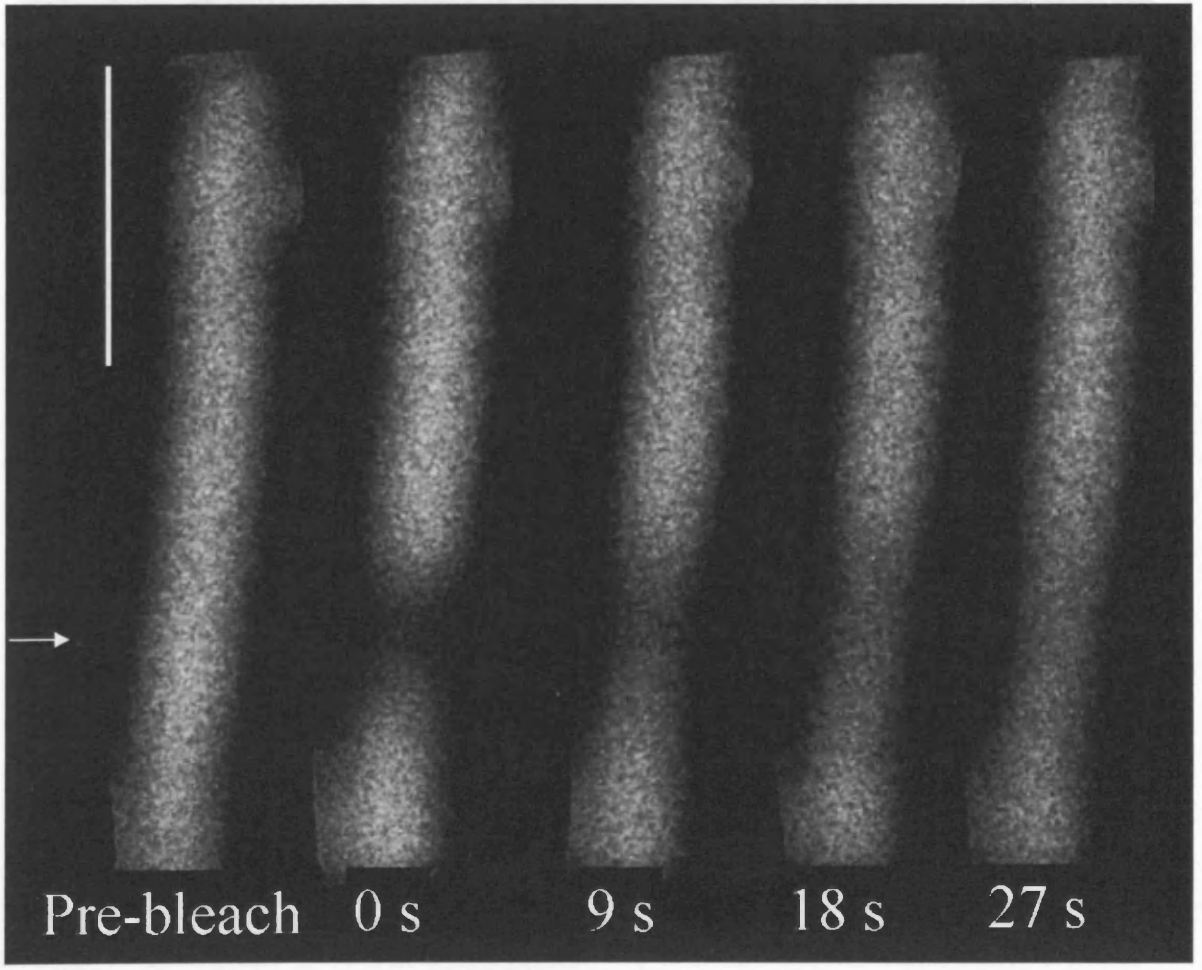


Figure 3.8. FRAP images of phycobilisome fluorescence of cells in growth medium. Scale bar = 5µm. Arrow denotes where the bleach will be most intense.

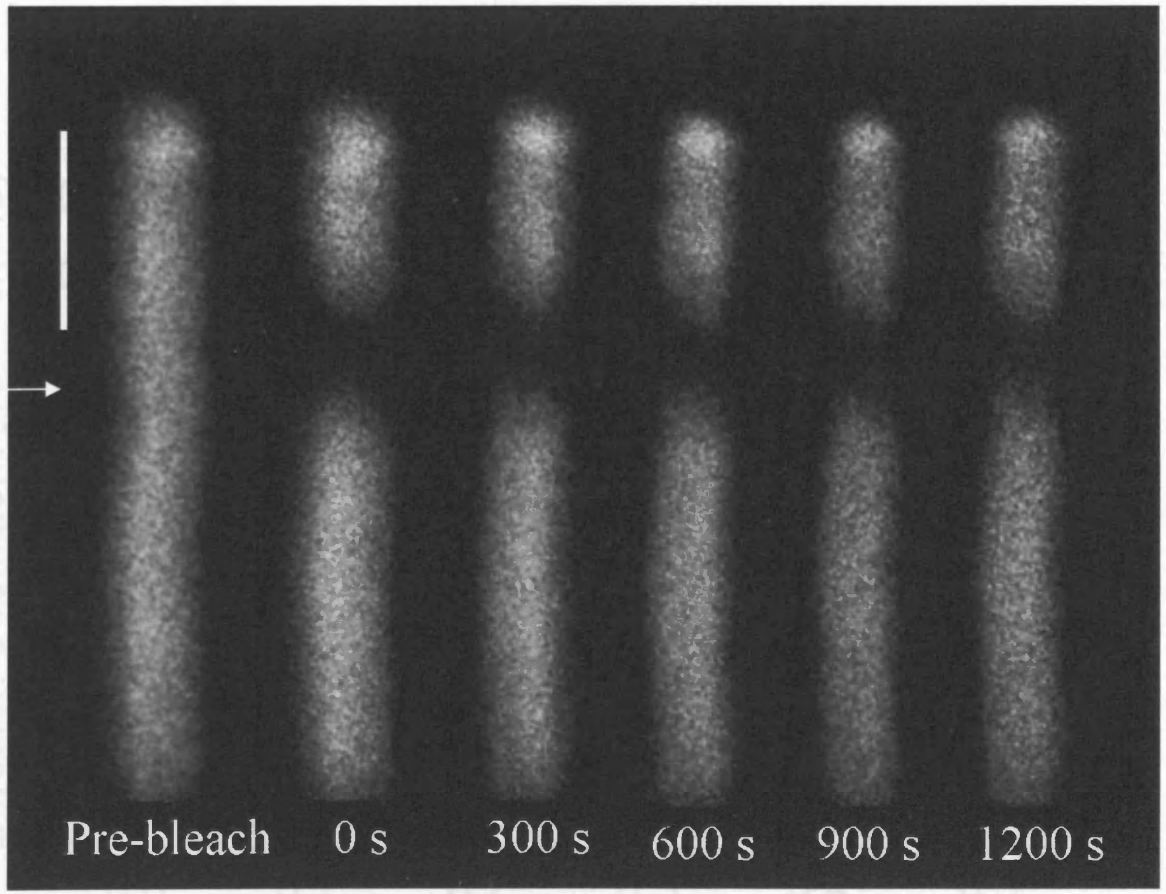


Figure 3.9. FRAP images showing phycobilisome fluorescence of cells in 0.5M phosphate.Scale bar = 5µm. Arrow denotes where the bleach will be most intense.

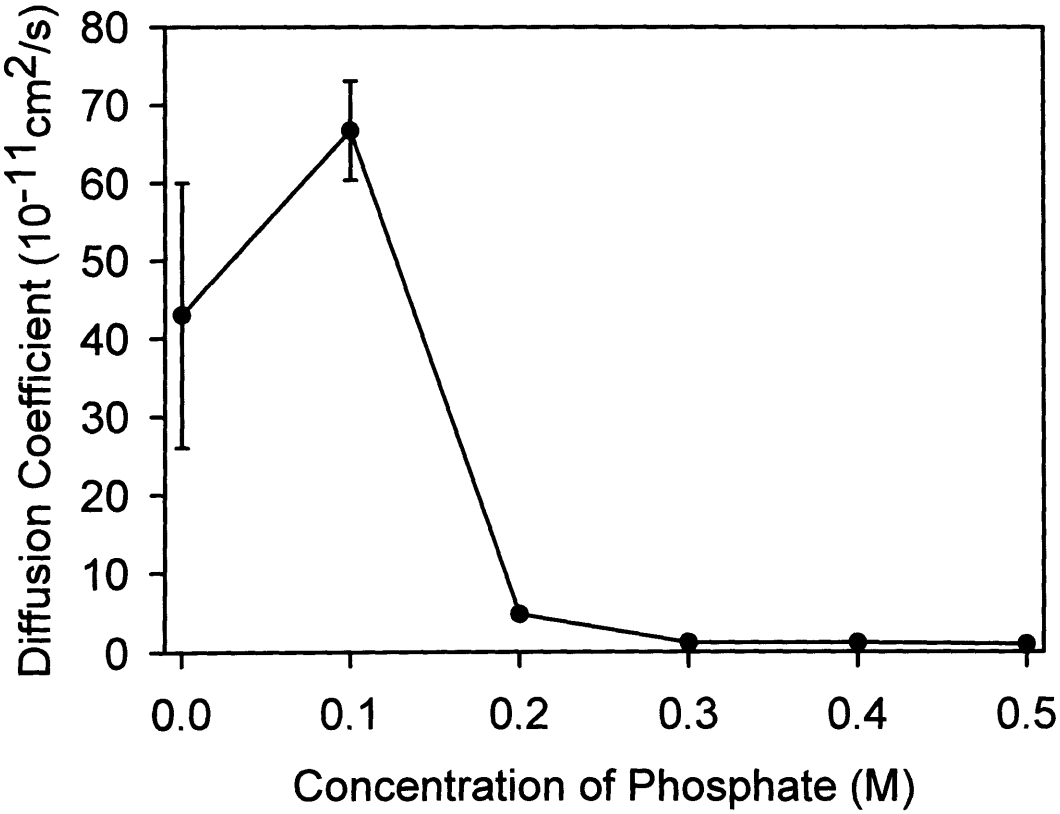


Figure 3.10. Effect of phosphate concentration on phycobilisome diffusion coefficient. n=6 for each concentration, with standard deviations given. Between 0.2M and 0.5M phosphate, standard deviations are too small to be visible.

3.5.3.2. Phycobilisome mobility of alternatively-adapted cells

Given that our results appear to show that the ability of sucrose to lock cells in state 1 is greater than in state 2 (figure 3.6), cells treated with 0.5 M sucrose following fixation in state 1 give significantly slower diffusion coefficients to those treated following adaptation to state 2, where phycobilisomes move at rates of 6.54(+/-0.35) x 10^{-12} $cm^2.s^{-1}$ and 4.66(+/-0.33) x 10^{-11} $cm^2.s^{-1}$ ($t=2.599$, $p<0.0019$) for state-1 and state-2-adapted cells. The effect is not significant using 0.2 M phosphate, the concentration at which there is most difference in fixation estimates for the two states. The diffusion coefficients are 3.98(+/- 0.31) x 10^{-11} $cm^2.s^{-1}$ and 4.39(+/-0.28) x 10^{-11} $cm^2.s^{-1}$ respectively.

3.5.4. Extraction of membranes with functionally intact phycobilisomes

Membranes with functionally coupled phycobilisomes can be extracted using SPCM buffer (Gantt *et al*, 1988). Since whole cells may be fixed in state using SPCM or other high osmotic strength buffers, it was reasonable to hypothesise that state fixed membranes could be obtained in the same way. Figure 3.11 shows that this is possible. However, it should be noted that over a period of 24 hours, membranes isolated with SPCM, and resuspended in SPCM or the less ionically strong 0.5 M phosphate buffers (data not shown) demonstrate increasing de-coupling and degradation of phycobilisomes, as shown in the increasing height and breadth of the peak at 650 nm respectively. Further, and consistent with data shown in chapter 3 on whole cells, there is less efficient energy transfer from the phycobilisomes to PSII (peak at 695 nm) when membranes are extracted using SPCM buffer.

Should a mobile model for state transitions hold true, there should be a difference in membrane topography of state 1-arrested membranes compared with membranes obtained from cells fixed in state 2.

Since its development, Atomic Force Microscopy (AFM) (Binnig *et al*, 1986) has become massively useful in the quest for elucidating the topography of various biological membranes (Drake *et al*, 1989). It will be particularly useful in determining the variable topography of thylakoid membranes, which are the most

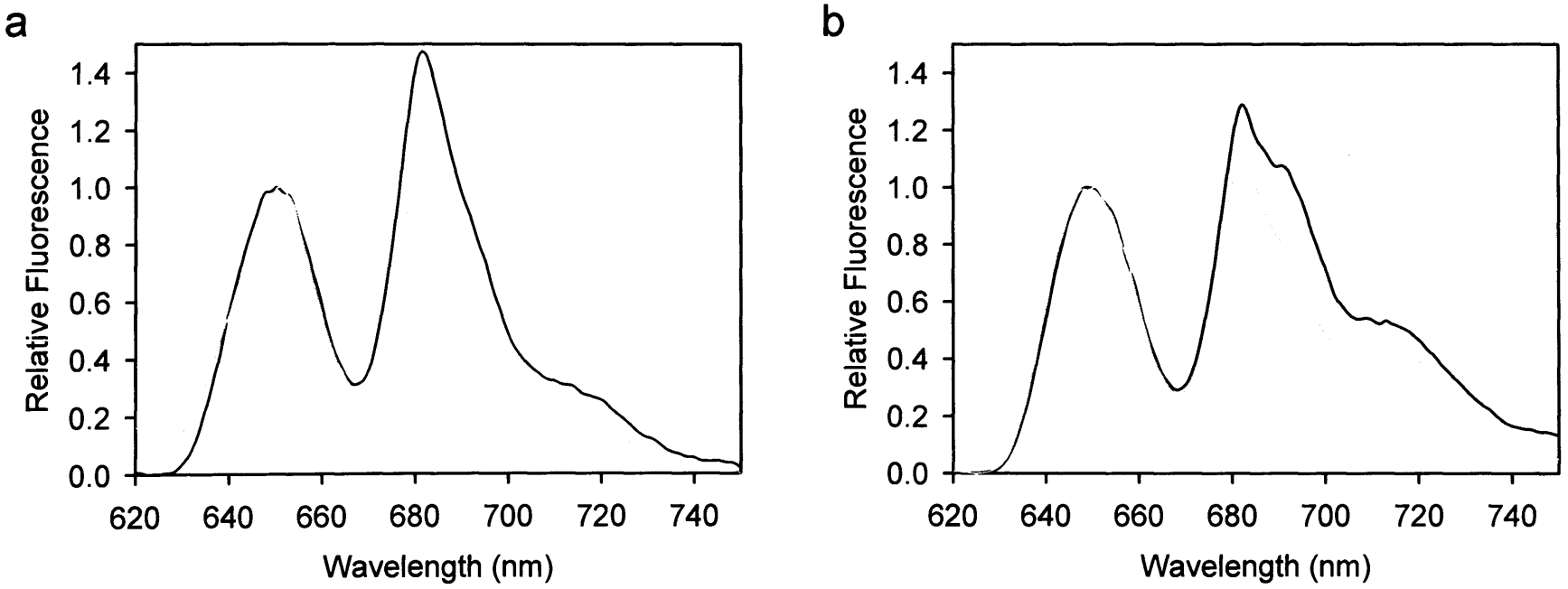


Figure 3.11. shows 77K fluorescence emission spectra for excitation of phycobilisomes at 600nm of light (black) or dark (grey) adapted *Synechococcus* 7942 wild type. A) Membrane preparations and B) whole cells, frozen at time t= 0. Spectra were obtained using excitation and emission slit widths of 5nm, and were normalised to phycocyanin fluorescence at 650nm.

abundant membrane type on Earth (Albertsson, 2001). Bahatyrova *et al* (2004) have recently shown thylakoid organisation at high resolution in purple bacteria using the technique. The advantage of the AFM over its predecessor is that as samples are not required to be conductive or maintained in a frozen, dried or vacuumed state, they can be observed under normal physiological conditions.

AFM does, however, rely on the ability of a silica nitride cantilever tip scanning the surface of a sample, and being repelled according to the charges it encounters on that surface. Whilst using high ionic strength buffers to screen out charge effects which could cause variability in height measurements are the norm, a buffer such as SPCM which is both sticky and highly charged results in the tip interacting with the sample by either being completely repelled or sticking to it and becoming contaminated, decimating the sample as it scans. Since the time between extracting the membranes and imaging them with AFM is in the order of hours, sample degradation would call into question the validity of images obtained. Therefore, they could not be determined as being accurate snapshots of topography at the point at which phycobilisomes were fixed to reaction centres upon addition of the buffer during the alternate illumination conditions.

No consistent patterns of membrane topography were recorded using the AFM technique for either wild type *Synechococcus* 7942 membranes with large phycobilisomes or the *Synechococcus* 7942 Δrod membranes which have small phycobilisomes lacking phycocyanin rods.

Phycobilisomes, the light harvesting complexes of cyanobacteria, diffuse upon the surface of the thylakoid membranes and distribute light energy to the reaction centres, PSI and PSII. Phycobilisome size and shape is variable with species (Bryant *et al*, 2001; Dilworth and Gantt, 2001; Gantt, 1981). The species used in this study, *Synechococcus* 7942, possesses hemidiscoidal phycobilisomes, with dimensions of approximately 70 nm x 50 nm X 35 nm (Sidler, 1994). Such large complexes should be easily detectable with AFM, and both their size and pattern of distribution may be analysable. There are no other known complexes which are located on the thylakoid membrane or protrude on the cytoplasmic side of this membrane which are of similar dimensions and could be confused with

phycobilisomes. ATP synthase is far smaller with different dimensions, protrudes a mere 10nm and is far less abundant.

A complication of this is that the interaction of PSI with phycobilisomes is more transient than that of the PSII-PBS complex, and over time, the phycobilisomes detach from the reaction centres and sit non-specifically on the membrane, the rate being asymmetrical for the differently adapted membrane preparations, and faster for those dark-adapted than light-adapted.

3.6. Discussion

It has previously been shown that the light harvesting apparatus of cyanobacteria, the phycobilisomes, are highly mobile complexes, diffusing on the thylakoid membrane and interacting with the two photosynthetic reaction centres distributing light energy (Mullineaux, 1997; Sarcina *et al*, 2001). Treatment of *Synechococcus* sp. PCC 7942 cells with buffers of high osmotic strength reduces the capability of the phycobilisomes to diffuse freely (figure 3.8). It is also known that photosystem 2 is immobile (Mullineaux, 1997; Sarcina *et al*, 2001) under normal conditions. Therefore, it is possible that the buffer is causing the phycobilisomes to adhere more strongly to the already restricted reaction centres. This is supported by the observation that phycobilisome-reaction centre complexes can be isolated intact using SPCM or glycine-betaine solution (Gantt *et al*, 1988; Li *et al*, 2004).

The inhibition of phycobilisome mobility by high osmotic strength buffer increases in a concentration dependent manner (figure 3.10), and cells immersed in 0.5 M phosphate give diffusion coefficients significantly lower than untreated cells. Restriction of phycobilisome movement is not specific to phosphate, as sucrose solutions over the same concentration range give comparable results. This suggests that the effect is osmotic. It is possible that by altering the osmotic balance within the cells that the phycobilisomes bind more tightly to the reaction centres owing to the absence of freely available water molecules. This theory is supported by data showing the inhibition to be reversible. When cells pre-treated with 0.5 M phosphate are spotted onto BG11 agar, phycobilisome mobility to un-treated levels

is restored. It can be proposed that the water removed from the cells upon immersion in phosphate is replenished by plating onto growth medium agar.

The short-term light adaptation mechanism, the state transition, occurs in cyanobacteria over a timescale of seconds to a few minutes, whereby illumination conditions which favourably excite one photosystem result in a greater proportion of excitation energy transfer to the other (Murata, 1969). In state 1, fluorescence from PSII is greater relative to PSI, the reverse being true for state 2. It is possible to "lock" cells into state 1 or 2 by immersing cells in phosphate buffer, inhibiting re-adaptation to the alternate state (Mullineaux, 1993). We show here that this is not species specific, as this fixation of state is observed using *Synechocystis* PCC 6803 in addition to *Synechococcus* sp 7942 and 6301. Once again, the inhibitory effect is not attributable to phosphate specifically, as these data are reproducible using sucrose solutions of similar concentration. Consistent with this, glycine-betaine has also been shown to fix *Spirulina platensis* cells in state 1 or 2 by drawing water out of the cells (Li *et al*, 2004).

In order for a transition to state 1 to occur, phycobilisomes must preferentially interact with PSII over PSI, the reverse being true for state 2. As such, the binding of the phycobilisomes to the reaction centres must be transient to enable flexibility of light harvesting depending on illumination conditions. Cells treated with 0.5M phosphate can be "locked" in state, possibly on account of phycobilisomes being unable to decouple from the reaction centres at their normal rates.

The critical concentrations required to completely inhibit the transition to state 1 are different for different osmotica. That phosphate buffers inhibit at a far lower concentration than KCl may in part be owing to the fact that while both the ions of the latter are smaller than phosphate, they may be capable of entering the cell; those that enter cause an osmotic gradient in the opposite direction. In the case of the phosphate buffers used, while the potassium ions will have a tendency to move, they will in fact be kept out of the cell due to electrostatic effects unless the phosphate ions can also enter. Since less will enter, the osmotic effect in the opposite direction will be lower, so more water will be drawn out of the cell.

While cyanobacteria have glucose transporters, they do not possess sucrose transporters, and so, sucrose is unlikely to permeate the cell in a similar manner, as it is also a much larger molecule than a potassium or chloride ion. Therefore, the osmotic gradient across the cell membrane is far larger in the presence of phosphate or sucrose compared with KCl, so more water may be drawn out of the cell at lower concentrations of buffer, lending weight to the hypothesis that the effect on phycobilisome mobility and state transition fixation is an osmotic one.

That sucrose requires a higher concentration than phosphate diffusion may be attributed to there being more ions in the phosphate buffer (potassium ions as well as phosphate ions) for every sucrose molecule at the same concentration.

Given that state transitions may be inhibited using a low ionic strength sucrose solution eliminates the possibility that the added ions (phosphate, for example) are simply inhibiting state transitions by virtue of an electrical effect.

The mechanism for the cyanobacterial state transition is the subject of great debate. The two main theories for how they are brought about are the spill-over model and the mobile model. The spill-over model postulates that phycobilisomes associate with PSII, and under illumination conditions where excitation to PSII is favoured, a certain amount of energy "spills over" into neighbouring PSI complexes (Biggins and Bruce, 1989).

The data presented here are consistent with the mobile phycobilisome model for state transitions, which requires that the light harvesting complexes diffuse freely along the thylakoid membrane, distributing light directly to each reaction centre under the appropriate conditions (Allen, 1992). It is apparently phycobilisome mobility which is inhibited by the addition of high osmotic strength buffers, slowing down and eventually preventing state transitions occurring. Chlorophyll in PSII is immobile even without the addition of the buffers, so an inhibition of spill-over, would, if anything, be a secondary effect.

The mobile model is supported by data obtained from different cyanobacterial mutants. It has been shown in the cyanobacterium *Spirulina platensis* that

phycobilisomes are four times more likely to be found interacting with PSI than PSII, refuting the postulates of the spill-over mechanism (Rakhimberdieva *et al*, 2001). Also suggestive of the mobile model is the observation that PsaL-lacking mutant in *Synechococcus* 7942 are capable of faster state transitions and give significantly larger phycobilisome diffusion coefficients, indicating the importance of the light harvesting apparatus' mobility to the state transition phenomenon (Aspinwall *et al*, 2004).

Other groups suggest that the mechanism of the cyanobacterial state transition is a combination of both the mobile and the spill-over hypotheses. Li *et al* (2004) suggest that as they have demonstrated that while locking phycobilisomes to the thylakoid membrane using a buffer of high osmotic strength inhibits state transitions, phycobilisome mobility is unaffected when state transitions are induced by a redox agent. Another suggestion that state transitions in these prokaryotes can be controlled by both redox signals as well as the location of the phycobilisomes relative to the reaction centres is supported by research on a state transition mutant of *Synechocystis* 6803, *rpa*C$^-$. Data shows that upon altering illumination conditions to attempt to bring about a state transition, a chlorophyll effect (increased PSII fluorescence relative to PSI in state 1 compared with state 2) is noticeable even though fluorescence from the phycobilisomes remains unchanged (Emlyn Jones *et al*, 1999). Further work must be carried out to determine whether RpaC really is affecting the mobility of the light-harvesting complex, possibly by making an inactivation mutant of the same gene in *Synechococcus* 7942, where FRAP measurements may be undertaken.

In conclusion, the FRAP data of phosphate-treated cells in conjunction with room temperature fluorescence transients and the 77K emission spectra showing state transition fixation over the same buffer concentration range provide evidence to suggest that phycobilisome mobility is critical for state transitions.

It is interesting that phycobilisome diffusion rates are so markedly different for alternately adapted cells treated with 0.5 M sucrose. That state 1-adapted cells give slower phycobilisome diffusion rates may be attributed to the phycobilisomes stronger affinity for PSII when in state 1. However, measurements are obtained

following lengthy adsorption times to agar, and indeed, in the light, so if this were the case, one would expect that in state 2, cells would have time to re-adapt to state 1 conditions. This is because fixation of state 2 is far lower at this sucrose concentration than fixation of state 1.

It is possible that a structural change occurs in the phycobilisomes when adapted to state 1, and that this is fixed upon addition of the buffer, causing them to dissociate from PSII complexes very slowly, thus giving slow diffusion rates. However, if cells have been adapted to state 2, they have weaker affinities for PSII. It is somewhat unlikely that they have as strong an affinity for PSI as a state1-type phycobilisome has for PSII, else this too would have resulted in small diffusion coefficients. That the state 2 phycobilisomes do not change into state 1-type phycobilisomes over the adsorption and duration of the recording of the measurement could be because the buffer inhibits such a change. Thus, state 2 phycobilisomes diffusing on the thylakoid membrane do not get fixed to PSII, and so, do not slow down in the same way as a phycobilisome configured to preferentially bind to PSII over PSI. In effect, this would mean that the buffer was acting to fix a state transition in two ways: first, by simply slowing down the phycobilisomes, and secondly, by preventing a structural modification.

A structural modification of a reaction centre cannot be ruled out either. This too, could have the effect of slowing down the phycobilisomes in state 1 but not in state 2. Further, this could be attributable to the presence or absence of a modifying molecule. Similarly, its attachment or removal may be inhibited by the presence of the buffer, inhibiting the change in mobility levels.

The fact that it is only at this concentration that differences in mobility are observed suggests that the overriding effect of the buffer is an osmotic effect on mobility of the light harvesting complexes. A secondary effect may be to inhibit a structural change.

Chapter 4. Inactivation of *rpa*C in *Synechococcus* sp. PCC 7942

Chapter 4: Inactivation of *rpa*C in *Synechococcus* sp. PCC 7942

4.1. Objectives

- To determine whether RpaC is required for state transitions in cyanobacterial species other than *Synechocystis* 6803
- To establish whether RpaC plays a role in the mobility of phycobilisomes on the thylakoid membrane

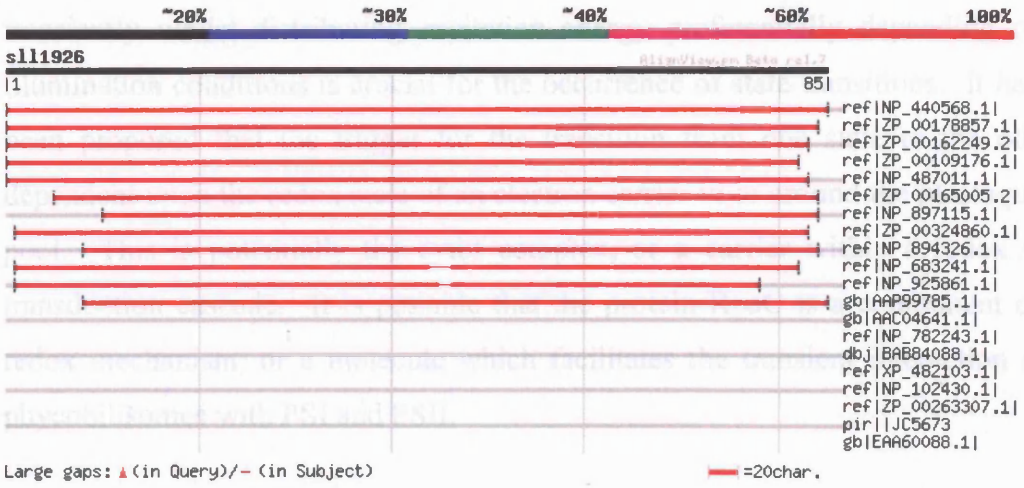
4.2. Introduction

The RpaC protein of *Synechocystis* 6803 has been identified to play a critical role in the ability of the cyanobacterium to carry out state transitions. A kanamycin resistant mutant of the relevant coding region of *Synechocystis* 6803, sll1926, was made which selectively inactivated the *rpa*C gene. Other than the inability of this mutant to conduct state transitions, it displayed no detectable phenotype different from wild type *Synechocystis* except for slower growth in very dim yellow light (Emlyn-Jones *et al*, 1999).

It is striking that the *rpa*C gene is conserved across several cyanobacterial species (see figure 4.1), suggesting that it plays an important physiological role. *rpa*C is not present in the completely sequenced, phycobilisome-possessing red alga, *Cyanidioschyzon merolae* 10D (Matsuzaki *et al*, 2004). BLAST searching of the Arabidopsis genome (http://mips.gsf.de/proj/thal/db/) did not yield sequences similar to *rpa*C, suggesting its lack of conservation in higher plants.

4.3. Rationale

The mechanism of state transitions in cyanobacteria has as yet not been resolved. Data presented in chapter 3 support the hypothesis that the light harvesting apparatus, the phycobilisomes, are required for state transitions. That is to say, their capability of diffusing between the photosynthetic reaction centres and interacting



Sequences producing significant alignments:	(bits)	Value
ref|NP_440568.1| unknown protein [Synechocystis sp. PCC 6803] gi...	167	6e-41
ref|ZP_00178857.1| hypothetical protein Cwat111001 [Crocosphaera...	130	9e-30
ref|ZP_00162249.2| hypothetical protein Avar020052 [Anabaena var...	119	2e-26
ref|ZP_00109176.1| hypothetical protein [Nostoc punctiforme]	119	2e-26
ref|NP_487011.1| hypothetical protein [Nostoc sp. PCC 7120] gi|2...	117	8e-26
ref|ZP_00165005.2| hypothetical protein Selo021275 [Synechococcu...	115	2e-25
ref|NP_897115.1| putative regulator [Synechococcus sp. WH 8102] ...	108	3e-23
ref|ZP_00324860.1| hypothetical protein Tery02005426 [Trichodesm...	106	1e-22
ref|NP_894326.1| hypothetical protein PMT0493 [Prochlorococcus m...	103	9e-22
ref|NP_683241.1| hypothetical protein tlr2451 [Thermosynechococc...	102	2e-21
ref|NP_925861.1| hypothetical protein gll2916 [Gloeobacter viola...	99	2e-20
gb|AAP99785.1| Uncharacterized membrane protein [Prochlorococcus...	96	1e-19

Figure 4.1. Conservation of *rpa*C across cyanobacterial species

Several sequences show homology to the *rpa*C translated sequence generated by probing with sll1926, the open reading frame corresponding to *rpa*C in *Synechocystis* 6803. This is a tblastn search of the amino acid sequence of RpaC. High "bit" scores and low "expected" values correspond to a low probability that such conservation between the search sequence and the BLAST-retrieved sequence occurs by chance. The sequences highlighted in red correspond to the *Synechocystis* 6803 and *Synechococcus* 6301 (closely related to *Synechococcus* 7942) sequences. Probing the JGI website (http://genome.jgi-psf.org/draft_microbes/synel.home.html) using the latter sequence derived from here extracted the *rpa*C gene insertionally inactivated in this chapter.

transiently whilst distributing excitation energy preferentially depending on the illumination conditions is crucial for the occurrence of state transitions. It has also been proposed that the trigger for the transition from one state to the other is dependent upon the redox state of an electron carrier in or around the plastoquinone pool. This is potentially the cytbf complex, or a carrier within a redox signal transduction cascade. It is possible that the protein RpaC is a component of this redox mechanism, or a molecule which facilitates the transient interaction of the phycobilisomes with PSI and PSII.

It is feasible to carry out FRAP experiments on *Synechococcus* 7942 owing to its elongated nature and the fact that it possesses rotational symmetry and regularly arranged thylakoid membranes. Such experiments, carried out on wild type cells *in vivo* have identified that phycobilisomes are able to diffuse on the thylakoid membrane with a mean diffusion coefficient of $3 \times 10^{-10} cm^2/s$ (Sarcina *et al*, 2001). In contrast, PSII is normally immobile (Sarcina *et al*, 2001) (except under certain stress conditions). An *rpa*C inactivation mutant in an organism where quantitative data of phycobilisome mobility is obtainable could help to pinpoint the mechanism of action of RpaC. An RpaC$^-$ mutant in *Synechococcus* 7942 would suggest that this conserved protein plays a key role in state transitions across cyanobacterial species, and since the complete sequence of *Synechococcus* 7942 has been deduced, the process of manipulation has become more accessible.

4.4. Results

4.4.1. Generation of an *rpa*C inactivation mutant in *Synechocystis* sp. PCC 6803

To confirm that sll1926 was important to the observation of state transitions in *Synechocystis*, an inactivation mutant of *Synechocystis* PCC 6803 was generated by transformation of the wild type cells using genomic DNA from the glucose tolerant mutant, where *rpa*C had originally been inactivated by insertion. Genotypic characterisation was achieved by PCR of the new mutant's genomic DNA using primers 1926F and 2005R (Emlyn-Jones *et al*, 1999), and segregation was confirmed (data not shown). Figure 4.2 shows the 77K fluorescence emission

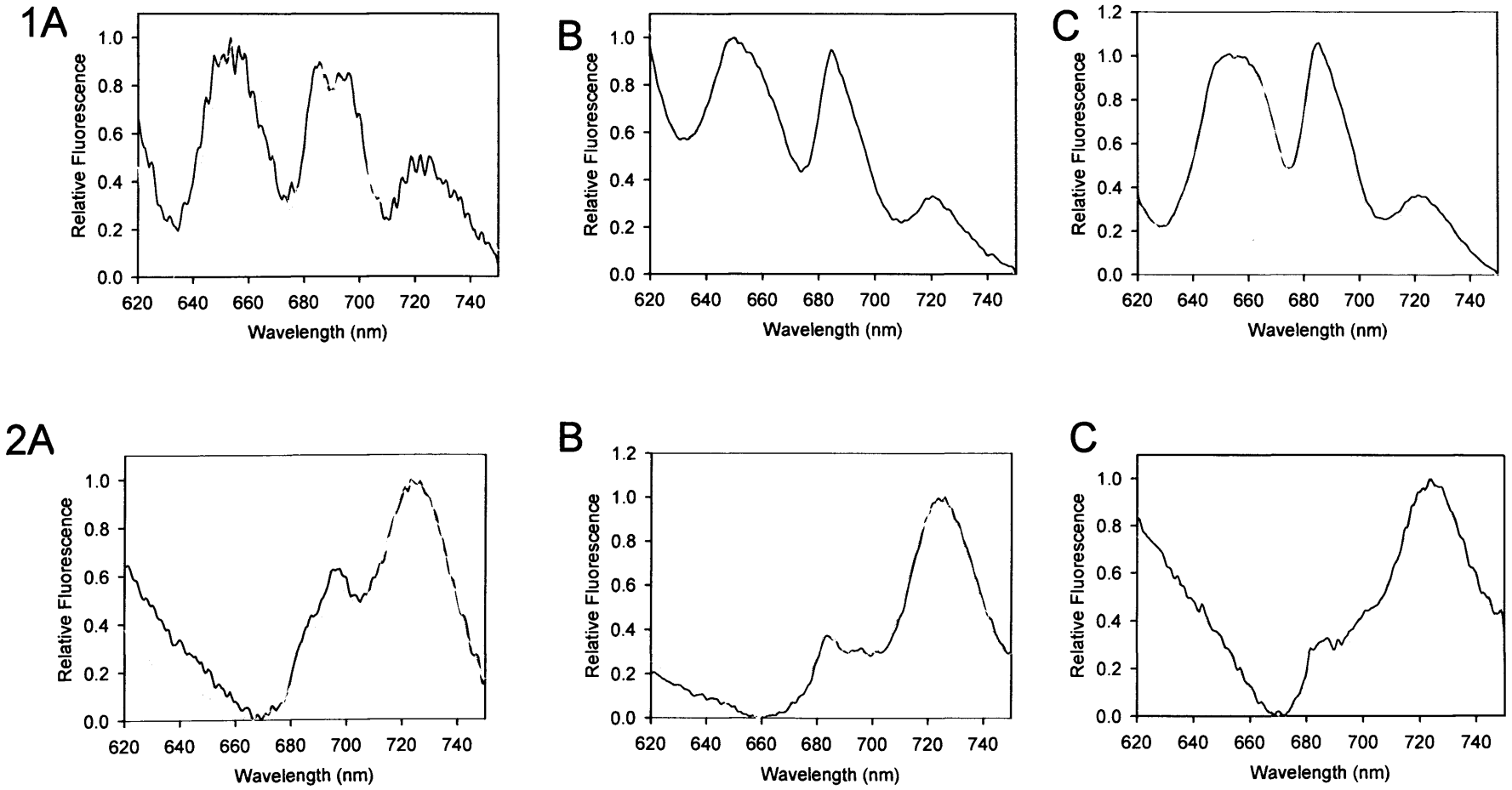


Figure 4.2. Confirming the state transition negative phenotype in *Synechocystis* PCC 6803 wild type (A), *Synechocystis* PCC 6803 *rpaC*$^{-}$ (B) grown under moderate illumination conditions. 1 and 2 show 77K spectra for excitation at 600nm (phycobilins), and 435nm (chlorphyll a) respectively. 1C and 2C depict the spectra for the glucose-tolerant mutant of the same species.

spectra for the mutant *Synechocystis* PCC 6803 *rpa*C$^-$, with the spectra for the wild type and glucose tolerant mutant also shown, for comparison. Unsurprisingly, the mutant in the non-glucose tolerant strain was unable to carry out state transitions as determined by fluorescence emission spectra. This confirms that in *Synechocystis* 6803, at least, the gene termed *rpa*C is required for state transitions.

4.4.2. An *rpa*C inactivation mutant in *Synechococcus* sp. PCC 7942

4.4.2.1. Generation of *Synechococcus* 7942 *rpa*C$^-$

Primers 1846F and 1846R were designed to amplify a region encoded by ORF481 from contig 52 of the *Synechococcus* 7942 genome sequence obtained from the United States Department of Energy Joint Genome Institute webpage, http://genome.jgi-psf.org/draft_microbes/synel/synel.home.html. This gene shows a degree of sequence similarity to sll1926 of *Synechocystis* 6803. A BLAST search of Cyanobase (http://www.kazusa.jp.or/cyanobase) reveals that ORF481 shows 67% identity to sll1926.

ORF481 is located from position 474515-474829 in contig 52. The nearest neighbouring genes, ORF 480 and 482, read in the opposite direction, from positions 474349-473027 and 476553-475420 respectively. Therefore, the *rpa*C gene in *Synechococcus* 7942 is not likely to be part of an operon.

The 657bp PCR product containing *rpa*C was ligated into the *Xho*1/*Sma*1 site of pBSsk$^-$. The resultant construct was digested with Bsg1, and blunt-ended with mung-bean nuclease before the 1.2kb *Hinc*II fragment of pUC4K corresponding to a kanamycin resistance cassette was ligated into the region. The plasmid construct depicted in figure 4.3 and harvested from transformed *E. coli* was transformed into *Synechococcus* 7942 and kanamycin resistant colonies were picked and repeatedly re-streaked.

Mutant genomic DNA was extracted. A PCR using primers 1846F and 1846R should have amplified a region of approximately 1.85kb. Since the wild type gene is retained, it can be concluded that the mutation never reached homoplasmicity.

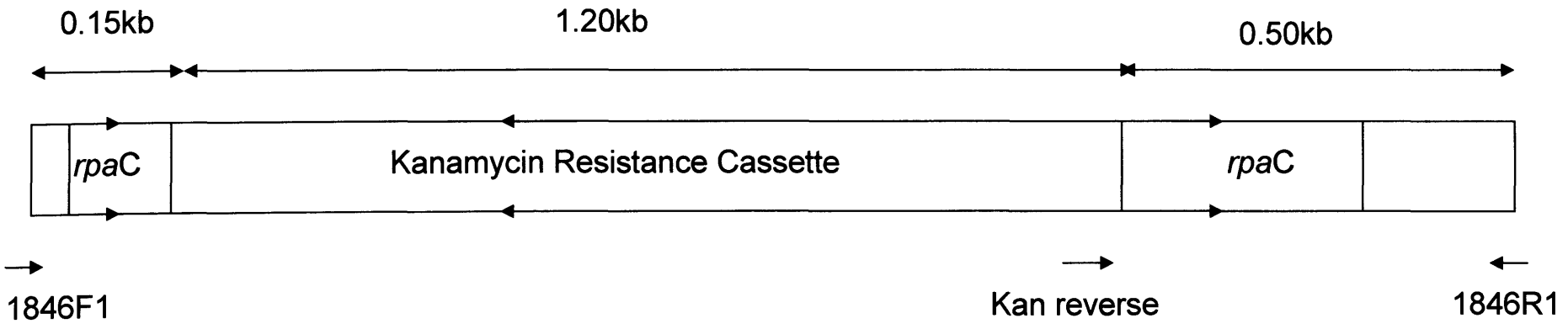


Figure 4.3. Diagram representing the *rpa*C inactivation construct in *Synechococcus* 7942.

To confirm that the kanamycin resistance cassette did indeed insert correctly, PCR was repeated using one of the original primers in conjunction with an internal kanamycin resistance sequence primer, kanReverse. Figure 4.4 is the gel photograph which confirms this.

The mutant would not segregate under a range of different illumination conditions including white light (high, moderate and low light intensity) and blue light. This suggests that the complete absence of RpaC is lethal in *Synechococcus* 7942 under all growth regimes used. It is unlikely that the mutant was the result of a single crossover event rather than a double, as 150bp either side is usually sufficient to drive homologous recombination (Conrad Mullineaux, personal communication).

4.4.2.2. mRNA transcript levels

Since the mutant did not segregate, semi-quantitative PCR was conducted in order to demonstrate that the mRNA transcription level had in fact been reduced in the mutant. A known concentration of total RNA was extracted from both mutant and wild type cultures grown under the moderate illumination conditions. Since cDNA from bacterial transcripts would be indistinguishable from genomic DNA sequences owing to the absence of intronic sequences, RNA was treated with DNase1 and repurified.

cDNA was made via reverse transcription using 1.5 μg RNA and primer 1846AR. The PCR carried out using primers 1846AF and 1846AR and $1/5^{th}$ of the products of the RT reaction. For control purposes, the gene coding for the D1 polypeptide of PSII, where the mutant was expected to give higher expression levels than the wild-type, was assessed in a similar way using primers psbA1R with reverse transcriptase and psbA1F and psbA1R for the PCR. Further, 7942rrna16SF and 7942rrna16SR were used to amplify the cDNA from the 16S ribosomal RNA transcript generated using the latter primer. The transcript levels were not predicted to differ in the wild type and the mutant. The gel photograph depicting the results is shown in figure 4.5.

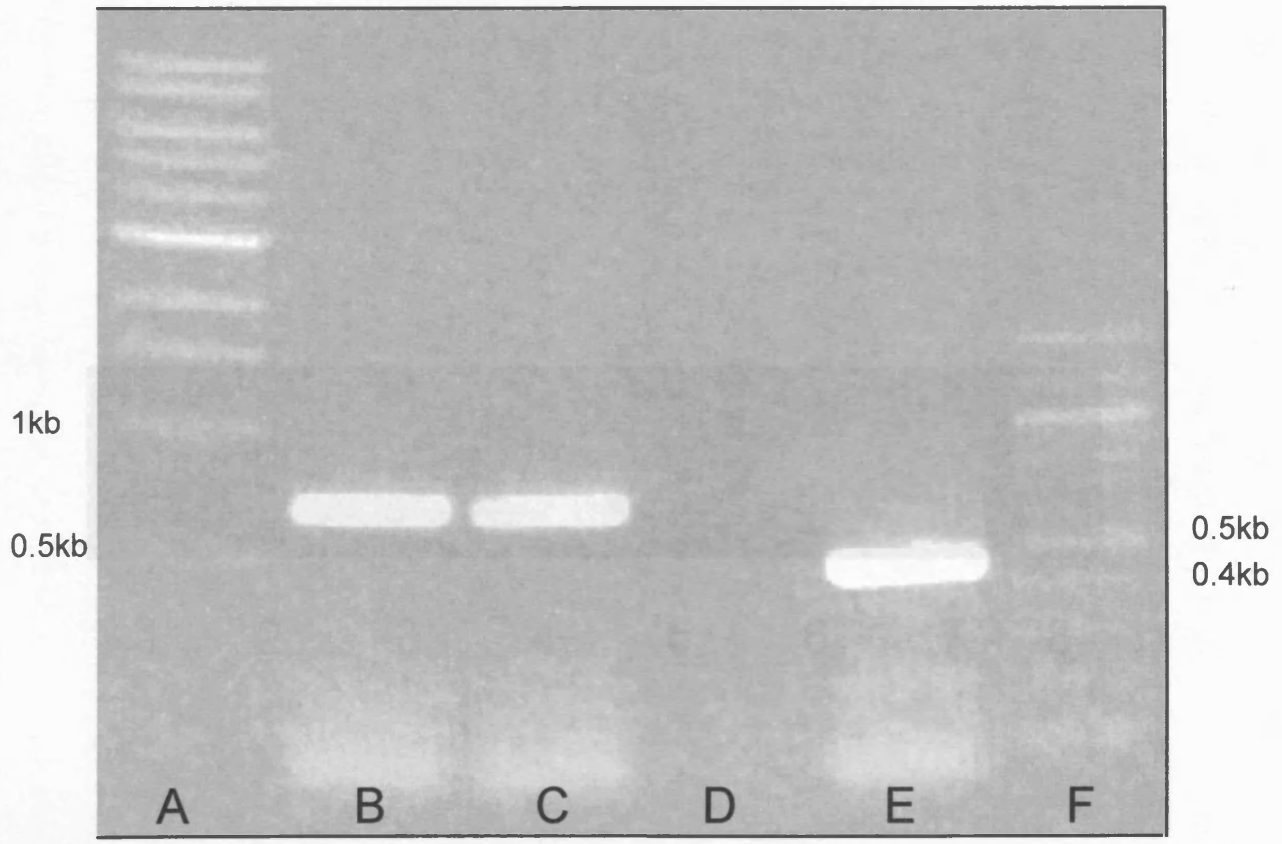


Figure 4.4. A gel photograph showing the sizes of the PCR products of *S.* 7942 wild type *rpa*C (B) and *S.* 7942*rpa*C^{-} (C) using the 1846F/1846R primer combination. Since the mutant is not segregated, PCR using kanReverse (an internal kanamycin resistance cassette primer) with one of the *rpa*C primers shows that the resistance cassette has been inserted. Lane D corresponds to PCR with 1846F/kanReverse (no amplification) and E shows the 400bp product obtained using 1846R/kanReverse, confirming that the cassette inserted antisense to the *rpa*C gene. Lanes A and F correspond to the NEB 1kb and 100bp ladders respectively.

Figure 4.5. mRNA transcript levels deduced via semi-quantitative RT PCR. Lanes 1 and 2 are wild type and mutant rpaC cDNA. Lanes 3 and 4 correspond to wild type and mutant psbAI. Lanes 5 and 6 are from 16S rRNA subunit of wild type and mutant cDNA. Lanes 7 and 8 show that no product will form from using rpaC specific primers if reverse transcriptase was not added to the RT reaction, showing that there was no DNA contamination in the RNA preparation.

A 150bp band corresponding to the psbA1 control indeed shows that D1 is up-regulated in the mutant, confirming observations that PSII/chlorophyll and PSII/cell is increased in *Synechococcus* 7942 *rpa*C$^-$ (see 4.4.2.3.3.). The 16S rRNA is shown to be transcribed at a far higher level in the mutant in than in the wild type. Given the latter result, it would be expected that the mRNA/total RNA is lower in mutant than wild type cells. It confirms, though, that the up-regulation of D1 transcription seen in the mutant is real, as the total mRNA in the mutant is lower than in the wild-type, so a more intense band from mutant cDNA suggests more D1 mRNA. Caution should be taken with the interpretation of this, as there are 3 psbA genes in this organism, expressed differentially under different illumination conditions. However, since the RNA was extracted from wild type and mutant cells grown to the same optical density under the same illumination conditions, this may not be considered to be a confounding effect.

The fact that a 200bp band corresponding to the presence of *rpa*C expression is evident in the mutant again confirms that the mutant is non-segregated. However, the expression of *rpa*C is lower in the mutant than the wild-type, suggesting that phenotypic differences between the wild type and the mutant are caused by an alteration in *rpa*C expression. The primers used are highly specific to the coding sequence of each gene, so it would be unlikely that the single product bands observed are PCR artefacts.

It is important to note that in *Synechocystis* 6803, *rpa*C mRNA transcript levels have only previously been detected when cells are grown under dim illumination conditions, and that the intensity of the signal from microarray data gets weaker as the illumination level is increased (Hihara *et al*, 2001). In *Synechococcus* 7942, where it is seen that *rpa*C is critical to cell viability, mRNA is detected strongly at higher illumination conditions than those used in the microarray study by Hihara *et al*. This adds further weight to the hypothesis that RpaC plays a far more crucial role in *Synechococcus* 7942 than *Synechocystis* 6803, where a knock-out is not only completely segregated, but is also shows no adverse phenotypes except under extremely low light growth regimes.

4.4.2.3. Pigment Content

4.4.2.3.1. Phycocyanin/Chlorophyll *a*

The absorbance of 400 nm to 750 nm light appears to be comparable for the wild type and mutant whole cells (not shown). The peaks at 625 nm and 678 nm were de-convoluted and used to estimate the phycocyanin and chlorophyll content in the cells using the formulae of Myers *et al* (1980), and cell numbers were quantified by counting using a haemocytometer. Slight differences in phycocyanin and chlorophyll per cell were not significant. Overall, the length of mutant cells was longer, and if this was taken into account, an estimate for pigment content per micron of cell may be obtained. Again, there is no significant difference between wild type and mutant cells (table 4.2).

Table 4.1. Summary of pigment content (number of molecules)

	Synechococcus 7942	
	Wild type	*rpa*C$^-$
Phycocyanin/cell	$(6.3 \pm 0.3) \times 10^7$	$(7.9 \pm 2.6) \times 10^7$
Chlorophyll/cell	$(1.0 \pm 0.1) \times 10^8$	$(1.3 \pm 0.4) \times 10^8$
Phycocyanin/Chlorophyll	0.63 ± 0.05	0.59 ± 0.01
PSII/cell	122000 ± 6000	206000 ± 9000
PSI/cell	420000	490000
PSI/PSII	3.4	2.4

Table 4.2: Results of t-test comparing wild-type *Synechococcus* 7942 with *Synechococcus* 7942 *rpa*C$^-$

PC/Chl	PC/μm	Chl/μm
$t = 1.27$, $p < 0.27$	$t = 0.70$, $p < 0.52$	$t = 0.58$, $p < 0.59$

4.4.2.3.2. Photosystem I

PSI content in the mutant is similar to that recorded in wild type cells (see table 4.1) using a flash photo-oxidation technique measuring absorbance of light at 703 nm

(by P700) (see 2.8.3 for details). PSII is fully functional in the mutant. This was estimated by subtracting absorbance values for the P700 estimation of cells at known chlorophyll concentration before and after the addition of DCMU. DCMU blocks electron transfer through PSII. Using this principle, the proportion of active PSII can be estimated by recording the absorption differences.

4.4.2.3.3. Photosystem II

Total PSII content (per cell and per chlorophyll) was deduced by an atrazine binding assay (where atrazine binds to the Q_B site of PSII) (see 2.8.4 for details). It appears that PSII is twice as high per mutant cell as in the wild type (see table 4.1). This would be expected if light harvesting to PSII was impaired by the downregulation of RpaC, as the cells would respond by up-regulating the synthesis of that reaction centre. PSII synthesis may also be increased if the absence of RpaC causes damage to PSII in some way.

4.4.2.3.4. Ratio of PSII/PSI

It is possible to estimate the PSII/PSI ratio from 77K fluorescence emission spectra for excitation of chlorophyll at 435 nm. The fluorescence ratio from PSII/PSI in the mutant is observable at 0.8, and the in wild type, at 0.4. This is consistent with there being no measurable difference in PSI content, and with there being an increased amount of PSII in the mutant (table 4.1).

4.4.2.4. Cell length

50 cells were measured along their long (Y) axis. The average length of wild type cells of *Synechococcus* 7942 was estimated to be 8.35±3.52 μm. Conversely, the mean length of the down-regulated mutant of RpaC was estimated to be 11.79±6.54 μm. Mutant cell length was significantly different from that of wild type ($t = 3.66$, $p<3.79\times10^{-4}$). This is depicted in figure 4.6.

4.4.2.5. Growth rates

Table 4.3 depicts the doubling times of wild type and mutant cells grown in a shaking illuminated incubator. The data presented are averaged from three different

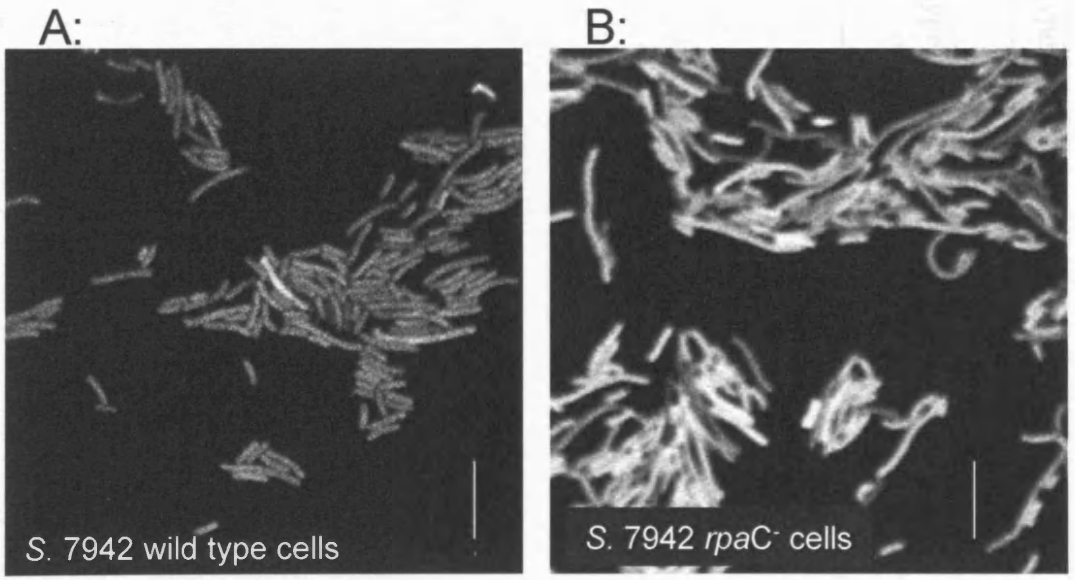


Figure 4.6. Comparing the lengths of wild type and mutant *Synechococcus* 7942 cells. 5μm scale bar

Table 4.3. Summary of Growth Experiments

Strain	Illumination Conditions	Mean doubling time (hours)
Wild type	High	9 ± 3
rpaC$^-$	High	18 ± 6
Wild type	Moderate	26 ± 9
rpaC$^-$	Moderate	24 ± 13
Wild type	Low	35 ± 7
rpaC$^-$	Low	45 ± 5
Wild type	Blue	71 ± 6
rpaC$^-$	Blue	74 ± 8

cultures, with standard deviations also provided. While no clear differences are observed under most conditions, the RpaC knock-down cells are disadvantaged over those of the wild type under high light. Care was taken to attempt to drive the mutant to segregation using different concentrations of kanamycin, as a non-segregated mutant may have been expected to express less kanamycin than is essential, causing a suite of phenotypes. However, doubling times were not significantly different across the range of antibiotics used (25, 50 and 100 μg/ml) (data not shown), so the table gives doubling times for cells grown in growth medium supplemented with the standard 50 μg/ml.

4.4.2.6. Oxygen evolution

Oxygen evolution was recorded for cells suspended to a known chlorophyll concentration. There does not appear to be any difference between the wild type and mutant cells in their ability to evolve oxygen in the light ($t=0.26$, $p<0.80$). Figure 4.7 demonstrates that the mutant and the wild type give comparable values for oxygen evolution under minimal and saturating red light intensities, is not suggestive of the mutant having a larger amount of active PSII, but the data do confirm that electron transport is occurring and that cells are capable of oxygen evolution. Cells had been grown under moderate illumination conditions. The readings are widely variable from day to day and culture to culture, and so, the standard deviations are large and differences cannot be deduced. The large degree of supposed heterogeneity in the extent of segregation may account for this, as measurements for the mutant are from cultures of cells which are likely to vary in the amount of wild type gene present. Since PSII synthesis is known to be increased in the mutant, it is possible that there is a certain amount of damaged and inactive PSII present in the membrane. Energy transfer to PSII may be defective in the mutant, so it is unsurprising that synthesis of PSII is increased relative to PSI in order to compensate for the deficiency and redress the balance.

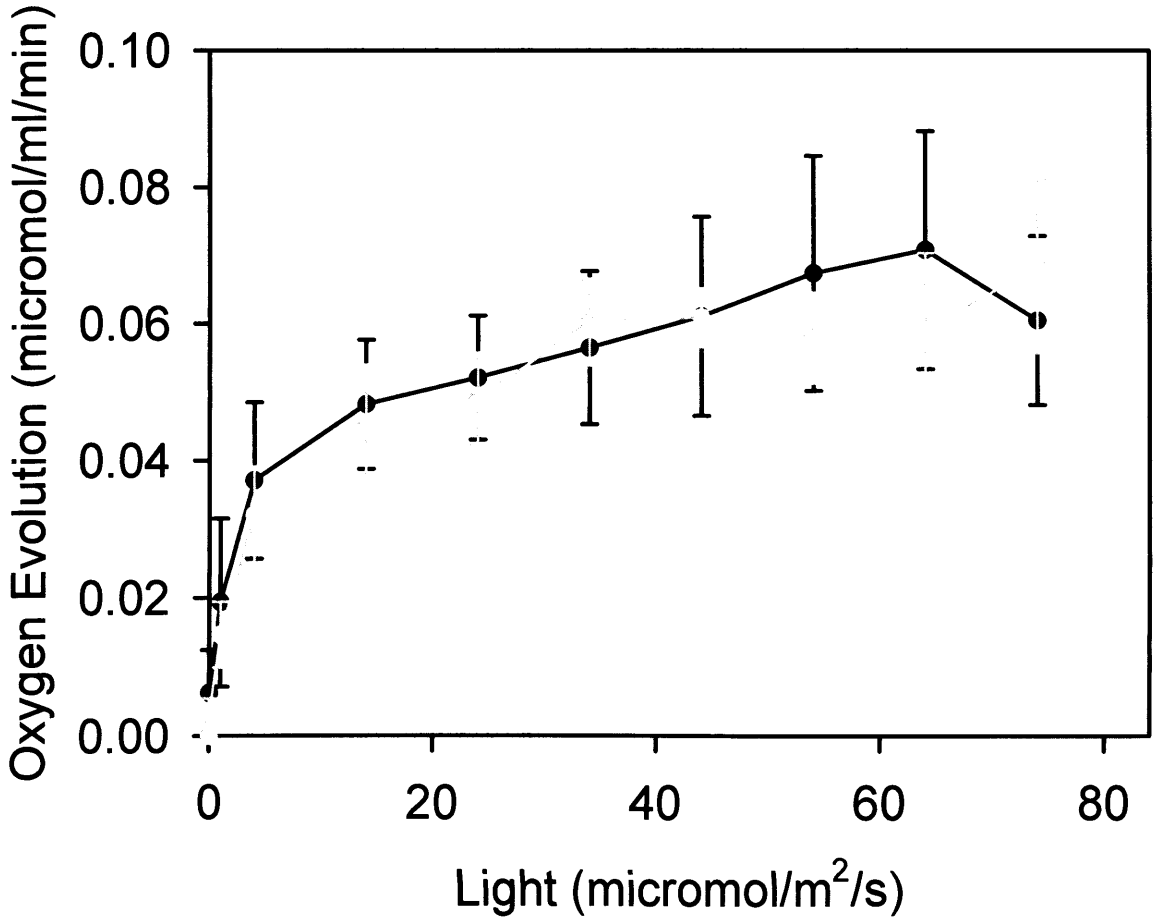


Figure 4.7.Oxygen evolution of wild type *S.* 7942 (black) and *S.* 7942*rpa*C^{-} (grey) using red wavelength illumination under increasing light intensities. Cells had been cultured under mid illumination conditions.

4.4.2.7. *Synechococcus* 7942 *rpa*C$^-$ and state transitions

In order to determine whether *Synechococcus* 7942 *rpa*C$^-$ was capable of carrying out state transitions, 77K fluorescence emission spectra were obtained, for excitation of phycobilisomes (600 nm) and for that of chlorophyll (435 nm) for cells grown in different light regimes. The results are shown in figures 4.8 and 4.9 respectively. The corresponding wild type spectra are provided for comparison (1a-c), the black trace referring to cells which have been light adapted, and the grey, dark adapted.

The emission spectrum for chlorophyll excitation of the mutant (2a-c, figure 4.9) is similar to that of wild type. In this case, the mutant behaves in a similar manner to that observed with the *rpa*C inactivation mutants generated in *Synechocystis* 6803. There are, however, marked differences with respect to energy transfer from the phycobilisomes to PSII in the *Synechococcus* 7942 knock-down mutant (2a-c, figure 4.8). The emission spectrum generated by this mutant suggests that a state transition of some sort is occurring, as there appears to be greater energy transfer to PSI relative to PSII where cells have been dark-adapted, compared with when a red light incubation has been employed. Interestingly, the shoulder characteristically observed at 685 nm, corresponding to the energy transferred to PSII by phycobilisomes in wild type cells is consistently smaller in the mutant. This was particularly noticeable when cells were frozen following the adaptation to state 1.

Figure 4.8 (2a-c) shows that the mutant is carrying out state transitions. It is notable that the 77K emission spectra show this under both a moderate illumination growth regime as well as when cells are grown in low intensity white light. It has previously been noted that state transitions in *Synechocystis* 6803 are reliably observed under conditions of dim illumination (Mullineaux and Emlyn-Jones, 2005), but they have also been observed at higher light intensities (this thesis, chapter 5). Since energy transfer to PSII appears to be defective, perhaps the observation of state transitions is to compensate for this, and therefore maximise light harvesting.

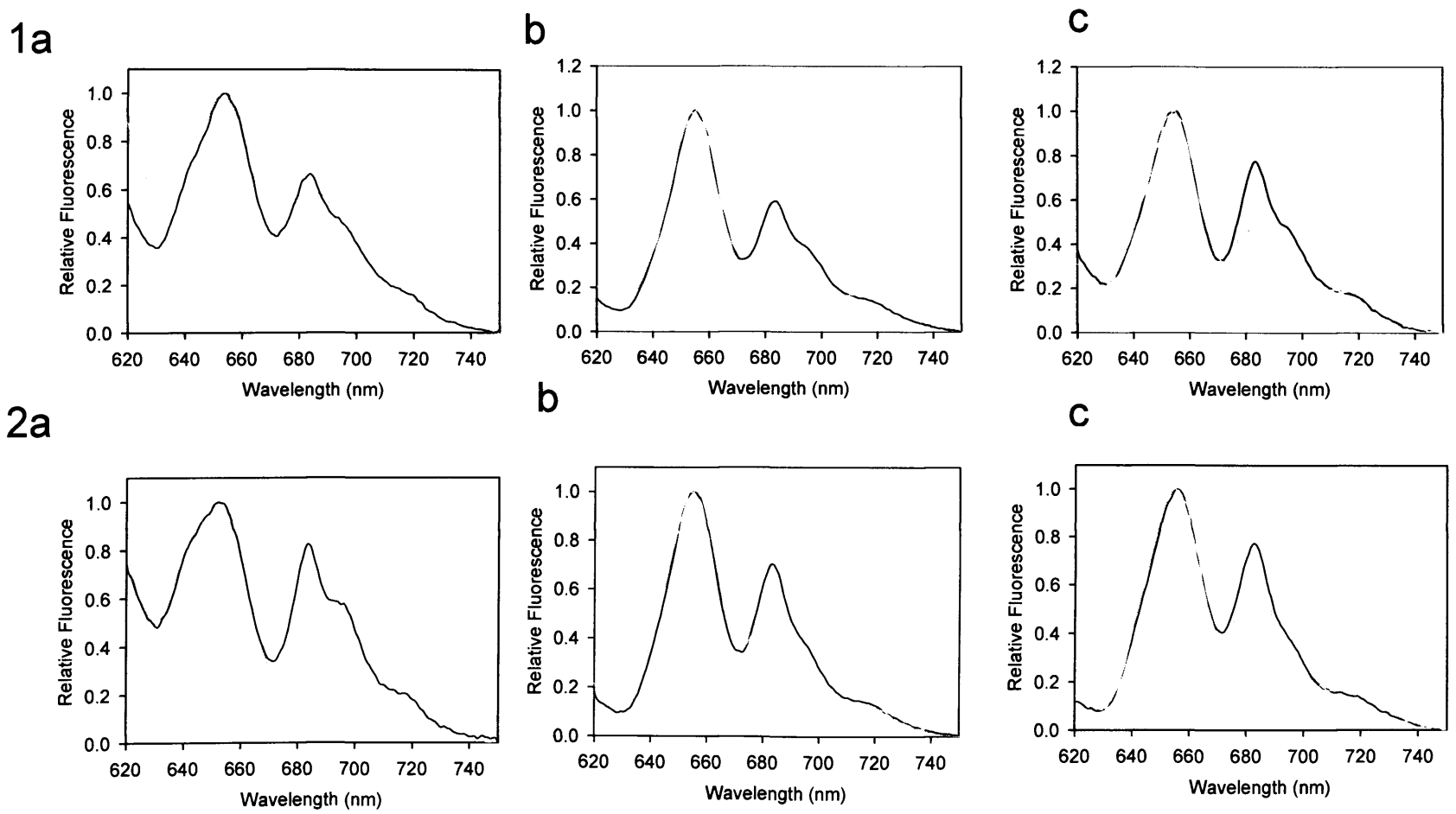


Figure 4.8. 77K whole cell fluorescence emission spectra for excitation of phycobilisomes at 600nm of light (black) or dark (grey) adapted *S.* 7942 wild type (1) and *rpa*C$^-$ (2) under high, mid and low illumination growth conditions (a, b and c respectively). Spectra were obtained using excitation and emission slit widths of 5nm, and were normalised to phycocyanin fluorescence at 650nm.

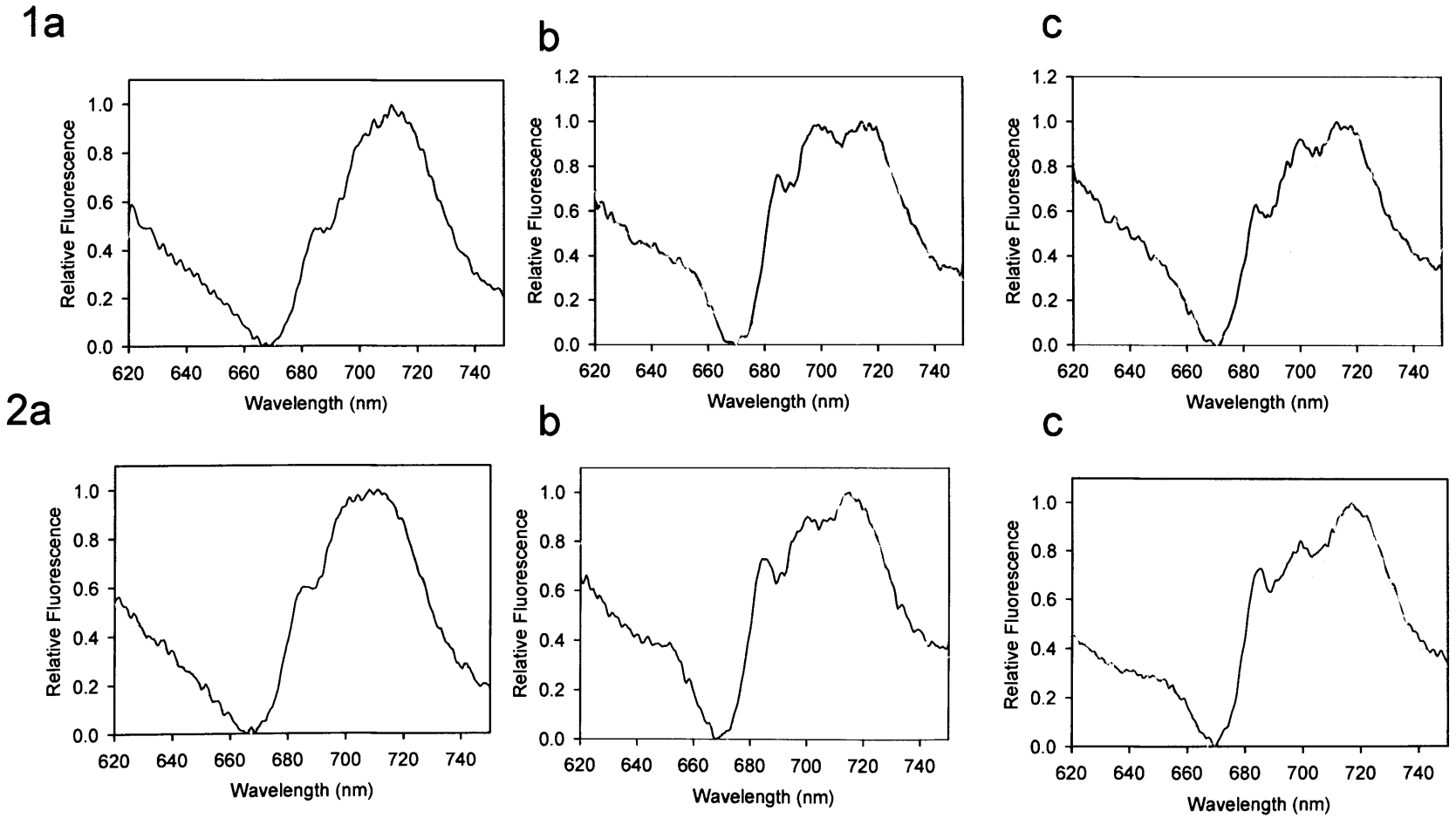


Figure 4.9. 77K whole cell fluorescence emission spectra for excitation of chlorophyll at 435nm of light (black) or dark (grey) adapted *S* .7942 wild type (1) and *rpa*C$^-$ (2) under high, mid and low illumination growth conditions (a, b and c respectively). Spectra were obtained using excitation and emission slit widths of 5nm, and were normalised to chlorophyll a fluorescence at 720nm.

Room temperature fluorescence emission time-courses are presented for the wild type and mutant in figure 4.10 (1a-c and 2a-c, respectively). If cells are carrying out state transitions, incubating cells that have been dark adapted for 5 minutes with red wavelength illumination results in a slow rise in fluorescence after an initial drop caused by opening of PSII centres. The reverse should occur when the light source is removed. The data in figure 4.10 support the 77K findings which show the mutant to be conducting state transitions under all illumination conditions as under all regimes tested, PSII fluorescence is increased upon red-light illumination and decreased upon state-2 induction in the dark. This is not different from the wild type, which is also state transition-competent using the same growth regimes. While it cannot be discounted that small quantities of *rpa*C are causing the observed state transitions, it is perhaps more likely that *rpa*C is not, as originally thought, a gene with the specific role of catalysing state transitions.

4.4.2.8. Phycobilisome mobility

A FRAP experiment to deduce whether there was any observable difference between the phycobilisomes in the mutant compared with wild type *Synechococcus* 7942 was carried out. Mutant cells give higher values for phycobilisome diffusion, 1.02(±1.20) $x10^{-9}cm^2s^{-1}$as compared with 4.80(±2.11) $x10^{-10}cm^2s^{-1}$ ($t=1.386$, $p<0.184$). This is not significant, but owing to the large degree of heterogeneity in segregation of mutant cells, differences may be masked. Mutant cells are much longer, denoting that they are under significant stress. This has previously been observed in other studies with this species (Aspinwall *et al*, 2004; Sarcina and Mullineaux, 2004). Longer cells may be more stressed on account of being more segregated for that mutation. It is not possible to check this, as it was not possible to separating cells according to size across a sucrose density gradient sufficiently well to perform PCR from DNA extracted from separated cultures. Phycobilisome mobility data alone are therefore not sufficient to support or refute whether RpaC is involved in the binding of the light harvesting apparatus to the reaction centres.

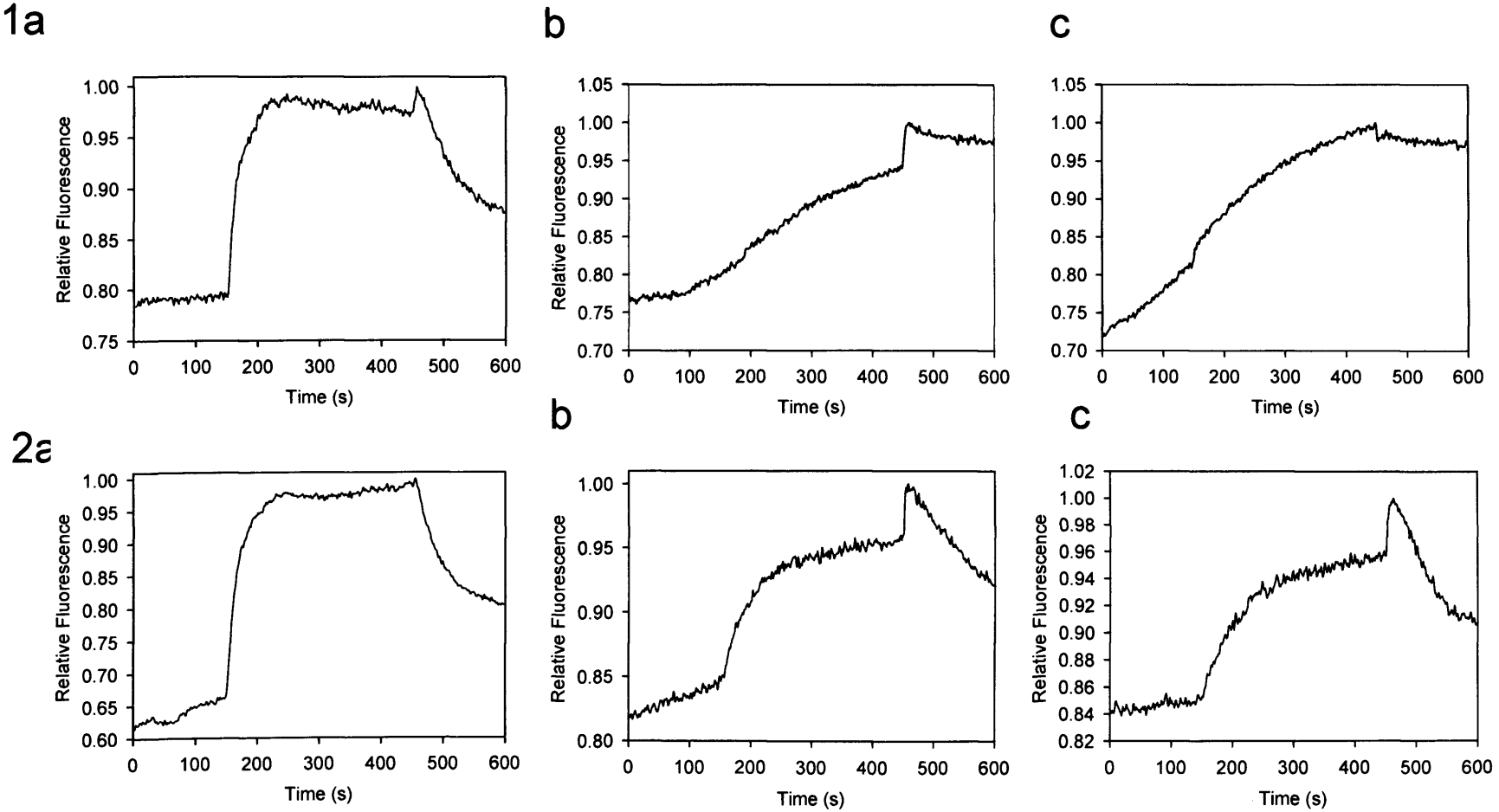


Figure 4.10. Room temperature time-course, for excitation of phycobilisomes at 600nm and emission at 680nm, corresponding to PSII. Traces presented are for *S.*7942 wild type (1) and *rpa*C^{-} (2) under high, mid and low illumination growth conditions (a, b and c respectively). Spectra were obtained using cells concentrated to 5μM chlorophyll, with excitation and emission slit widths at 10nm and 5nm. Red illumination was provided between 150s and 450s, otherwise cells received dim yellow illumination.

4.4.2.9. Mobility of PSII

Consistent with previous work (Sarcina *et al*, 2001), PSII in wild type *Synechococcus* 7942 does not diffuse. PSII does not diffuse in the mutant cells except under conditions where cells have been grown in high light intensity. Mean diffusion coefficients for PSII chlorophyll in the mutant were recorded at 7.65 (±4.44) $x10^{-10}cm^2s^{-1}$. Recent work on photo-inhibited wild type cells shows that a proportion of the cell's PSII may be mobile (Sarcina and Mullineaux, 2004; Sarcina *et al*, 2005), so the observed diffusion of PSII in *Synechococcus* 7942 *rpa*C$^-$ may be attributed to cells being under stress and the induction of the PSII repair cycle.

4.4.2.10. State transition fixation with high osmotic strength buffers

*rpa*C$^-$ mutants of *Synechocystis* 6803 appear to be locked in state 1 (Emlyn-Jones *et al*, 1999), and the inability to carry out the state transition appears to be on account of some aspect of phycobilisome association, as 77K fluorescence emission spectra where chlorophyll *a* has been excited (at 435 nm) do not appear different from the wild type.

FRAP data on *Synechococcus* 7942 wildtype cells fixed in state by 0.5 M sucrose show that phycobilisome diffusion rates are dependent upon the light-state cells were adapted to prior to fixation (Chapter 3 of this thesis; Joshua and Mullineaux, 2004). This result may indicate a conformational change in some of the light harvesting complexes. Alternatively, and in perhaps a more likely scenario, the fact that the phycobilisomes bind more effectively to one reaction centre (PSII) than the another (PSI), means that the light harvesting complexes would be slowed down in state 1 due to being more retarded by the preferential binding to PSII.

If the mutants are unable to make a conformational alteration (possibly via covalent modification) of their phycobilisomes, or that the phycobilisome-PSII interaction is less strong than in the wild type, the phycobilisomes will have similar capability to diffuse, so it might be predicted that there would be no such asymmetry in phycobilisome mobility at 0.5 M sucrose, regardless of whether cells were light or dark adapted prior to treatment.

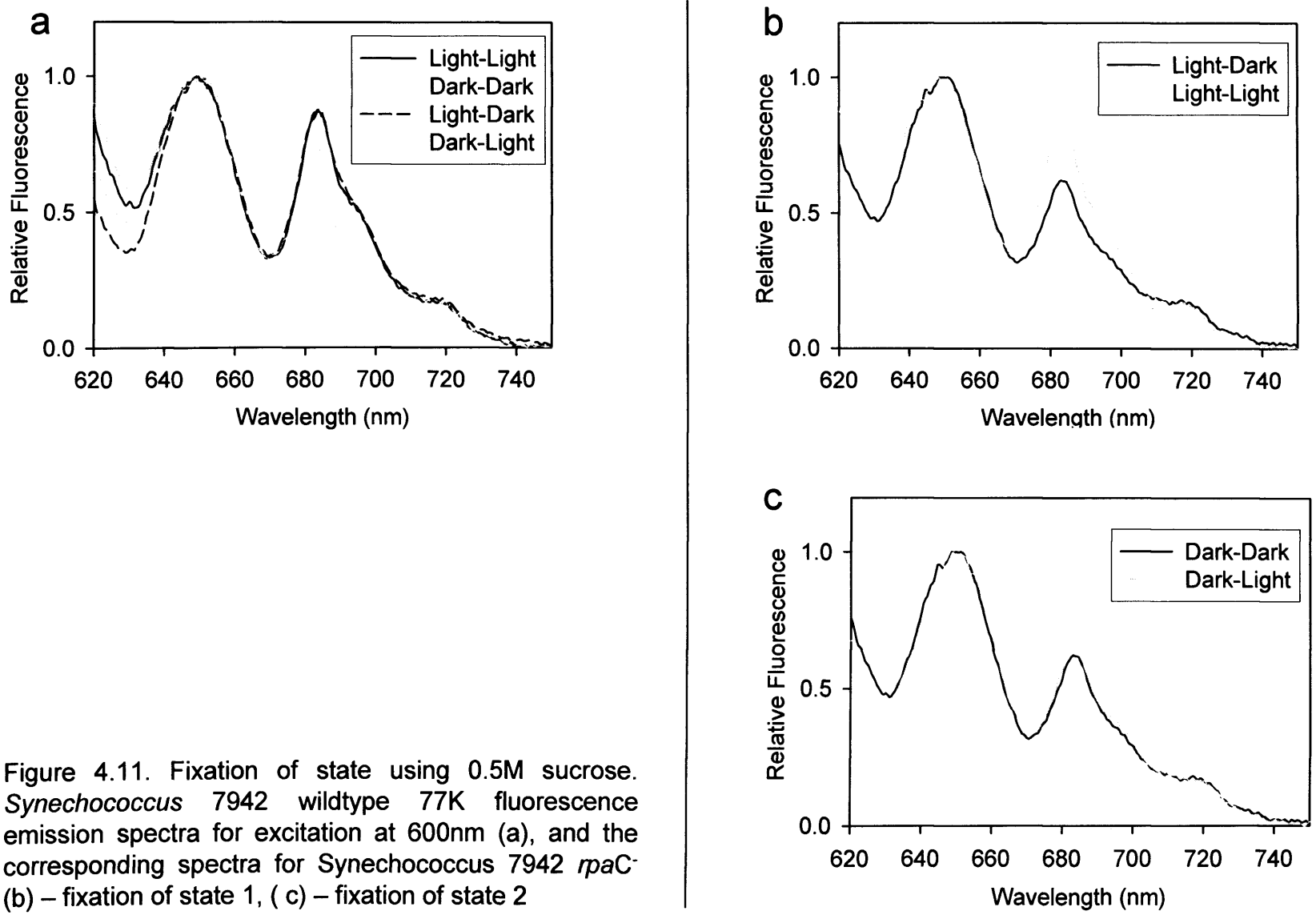


Figure 4.11. Fixation of state using 0.5M sucrose. *Synechococcus* 7942 wildtype 77K fluorescence emission spectra for excitation at 600nm (a), and the corresponding spectra for Synechococcus 7942 *rpa*C$^-$ (b) – fixation of state 1, (c) – fixation of state 2

Since it appears from low temperature emission spectra that phycobilisomes in the mutant of *Synechococcus* 7942 do not bind as effectively to PSII than the wild type, it may be that it might be harder to fix cells in state 1 using sucrose. Figure 4.11 shows the wild type and mutant fixation of state 1 and state 2 using 0.5 M sucrose. The estimate of state 1 fixation (see chapter 3 for details) using 0.5 M sucrose is 0.96±0.10, with state 2 presenting fixation estimates of 0.71±0.16. Therefore, perhaps surprisingly, there is no difference in fixation of state 1 between the wild type and the mutant. Instead, the difference exists in fixing state 2, with the mutant fixing phycobilisomes to PSI with greater ease than the wild type (0.2 M fixation, see chapter 3). This does seem to be consistent with 77K emission spectra which show that the mutant is generally more state 2-like, as the F685/715 is perpetually lower than in the wild type.

The mean phycobilisome diffusion coefficients for light-fixed mutant cells and dark-fixed ones treated with this concentration of sucrose are 4.09 (±0.24) x 10^{-11} $cm^2.s^{-1}$ and 3.13 (±0.16) x10^{-11} $cm^2.s^{-1}$ respectively. This difference is not significant, unlike data presented for 0.5 M sucrose-state-fixed wild type cells (chapter 3), where a clear difference was reported.

While there is no difference between the diffusion rates for state 2 fixed cells between the wild type and the mutant ($t=1.10$, $p<0.32$), mutant cells clearly exhibit faster phycobilisome diffusion when cells have been pre-adapted to state 1 and fixed with 0.5 M sucrose ($t=3.24$, $P<0.01$). This also applies using 0.2 M phosphate. This result supports the hypothesis that the phycobilisomes are not interacting as strongly with PSII in the mutant, and therefore may be resulting in reduced ability to transfer light energy to the reaction centre without causing damage.

4.5. Discussion

An *rpa*C knock-down mutant has been generated in a second cyanobacterial species, namely *Synechococcus* 7942. Down-regulation of the *rpa*C gene in *Synechococcus* 7942 confers the ability to conduct constitutive state transitions, a phenotype different from that observed in *Synechocystis* 6803, where inactivation of

*rpa*C nullified the ability to carry out state transitions (Emlyn-Jones *et al*, 1999). The gene is conserved across cyanobacterial species, suggesting its importance, and the observation that the mutation will not segregate points to its absence being lethal in *Synechococcus* 7942.

Mutant cells are considerably more elongated than wild type cells, suggesting that they are more stressed than the latter. This property has been reported previously in iron-starved *Synechococcus* 7942 cells expressing IsiA (Sarcina and Mullineaux, 2004). Further, *Synechococcus* 7942 *rpa*C$^-$ cells give slightly faster phycobilisome diffusion coefficients. We can propose that segregation of the mutation varies from cell to cell. Increased cell length correlates with increased diffusion of phycobilisomes, again suggesting that longer cells are more highly segregated for the deletion. However, since it has proved too difficult to separate out longer cells without contamination from the shorter ones, this cannot be confirmed by analysis of the genotypes or fluorescence properties.

The mutant generated in *Synechococcus* 7942 appears to have comparable pigment content, and functional phycobilisomes, PSII and PSI. It does have more PSII overall than the wild type, suggesting that the cells are compensating for the skew in light harvesting of the phycobilisomes to PSI even under conditions where state 1 is usually induced.

State transitions in cyanobacteria are known to be controlled by the redox state of an intersystem electron carrier associated with the plastoquinone pool (Mullineaux and Allen, 1990). It has previously been suggested that RpaC may play a role in signalling to prompt a state transition (Emlyn-Jones *et al*, 1999), but the fact that the mutant in *Synechococcus* 7942 would not segregate makes this unlikely. An alternative is that it may play a role in altering the structural properties of reaction centre-phycobilisome complexes. We investigated the latter using high osmotic strength solutions.

It has recently been shown using *Synechococcus* 7942 wild type cells that phycobilisome diffusion is a critical requirement for state transitions (see chapter 3 and Joshua and Mullineaux, 2004). This was achieved by arresting cells in state 1

or state 2 using phosphate buffers or sucrose solutions. State 1-adapted cells have significantly slower diffusion coefficients than those alternately adapted. This observation was reversed in the mutant, but state 2-adapted mutants yielded no significant differences in phycobilisome diffusion than the correspondingly treated wild type cells. Phycobilisome diffusion may be limited by immobile membrane components or by steric hindrance in the cytoplasm (Mullineaux *et al,* 1997). The data presented here (from fixation of cells in state 1) strongly indicate that down-regulation of RpaC carries implications for the structural coupling between the immobile PSII and the light harvesting complexes. This supported by evidence in a PSI-RpaC$^-$ double mutant strain of *Synechocystis* 6803 that energy transfer to PSII is hampered compared with a single PSI$^-$ mutant (Emlyn-Jones, 1999, PhD thesis).

Since we know that RpaC is a membrane-spanning protein, it is unlikely to alter the speed of the phycobilisomes by binding to them differentially under the different illumination conditions to catalyse a state transition. Thus, the other possibility that should be examined is that RpaC sits adjacent to PSII and assists in coupling the phycobilisomes to it. In its absence, there is nothing to aid the binding, resulting in

a) a greater number of phycobilisomes binding to PSI, accounting for our observation from the 77K fluorescence emission spectra that the majority of cells are in state 2;
b) faster phycobilisomes, as in the wild type, state-1 fixed cells diffuse much slower, suggesting that PSII-PBS coupling is stronger usually than PSI-PBS coupling.

In conclusion, it may be proposed that RpaC is not directly required for state transitions as was previously reported (Emlyn-Jones *et al*, 1999). Rather, it is likely that it is a factor which aids binding of phycobilisomes to PSII. A possible reason for its down-regulation yielding a different phenotype in *Synechocystis* 6803 (that is, arresting cells in a state 1-like phase), is that *Synechocystis* 6803 cells may be capable of increasing the expression of an alternate phycobilisome-PSII binding factor, increasing the intensity of association, and preventing the subtle regulation of light harvesting known as state transitions. *Synechococcus* 7942 is not particularly closely related, and may not have evolved a mechanism of coping with such a loss. It may also be attributed to the fact that *Synechococcus* 7942

phycobilisomes have only two APC core complexes compared with the three possessed by *Synechocystis* 6803.

Further work on this gene could include epitope tagging and subsequently fractionation experiments which may yield information about which complexes in the membranes it associates with. A GFP tagged RpaC would also be of use, in that FRAP experiments in *Synechococcus* 7942 could provide clues as to whether the protein is mobile, expressed at all (it is possible, though unlikely given its putative, well conserved transmembrane helices, that it mediates phycobilisome-reaction centre binding indirectly, as small mRNA) and associating with which complexes.

Chapter 5: Over-expression of RpaC in *Synechocystis* sp. PCC 6803

Chapter 5: Over-expression of RpaC in *Synechocystis* sp. PCC 6803

5.1. Objectives

- To generate an over-expression mutant of *Synechocystis* 6803.
- To determine if this mutant is capable of state transitions with a view to making a GFP-tagged RpaC mutant from the construct generated here.

5.2. Rationale

In *Synechocystis* 6803, the short term light adaptation phenotype of the state transition is known to require a small membrane-targeted protein of approximately 9kDa molecular weight. Inactivation of the gene *rpa*C obliterates the ability for *Synechocystis* 6803 to carry out state transitions, and the inactivation mutant is locked into light state 1 (Emlyn-Jones *et al*, 1999). It has been observed that state transitions generally occur when cells have been grown under conditions of low illumination (Mullineaux, 2004). It is therefore unsurprising that *rpa*C mRNA transcription levels decrease sharply as light intensities are increased above these low levels (Hihara *et al*, 2001). As yet, it has proved impossible to extract RpaC protein from wild type *Synechocystis* 6803 cells, even those grown in dim light. Therefore, while it would prove useful to generate a GFP-tagged mutant of RpaC, the chance of being able to detect the protein is diminished by its low level of expression (Hihara *et al*, 2001).

It was previously anticipated that RpaC acts as a component of a signal transduction cascade, and as such, may be capable of regulating phycobilisome association with one or the other photosynthetic reaction centre under varying illumination conditions in order to maximise light harvesting. Data presented in chapter 4 from a *Synechococcus* 7942 deletion mutant of *rpa*C suggests that the protein is not acting to reduce or increase the ability to bind or dissociate the phycobilisomes from the PSI reaction centres, but there is a strong suggestion that its down-regulation is having an impact on the ability to bind to PSII. Evidence for this is that the diffusion coefficients for the phycobilisomes are not significantly different from those in wild type cells, where each had been fixed in state 2, but is different if they had been state 1-fixed with a high osmotic strength buffer.

The trigger for state transitions in cyanobacteria has been determined to be the redox state of an electron carrier around the plastoquinone pool or the cytbf complex. The fact that biochemical analysis has suggested that RpaC has two membrane spanning domains in turn suggests that the protein is locked in the membrane, and we cannot discount that it may be signalling between PSI and PSII. Given that most electron carriers have obvious sequence motifs which are absent in *rpa*C, it is unlikely that RpaC could be the key electron carrier, especially in the light of the results presented in chapter 4, which suggest the protein is some kind of binding factor.

The over-expression mutant is to be generated by placing *rpa*C directly downstream of the promoter of *psb*A2. There are multiple copies of PsbA in the cyanobacterial genome which differ slightly in both sequence and expression level. PsbA codes the D1 protein of PSII. Given that it is present in multiple copies, it is not surprising that other groups have exploited this feature in order to generate over-expression constructs using the *psb*A2 promoter region (Lagarde *et al*, 2000), as *psb*A2 is dispensable in the presence of the other *psb*A genes. Since PsbA2 is generally being expressed continuously, would RpaC be expressed constitutively as well, regardless of the conditions the cells are being grown in? Does over-expressing the protein give the phenotype of constitutive state transitions, or might the mutant be more light sensitive and therefore more prone to photo-inhibition? The answers to the above questions will permit the design of a tag on RpaC.

5.3. Results

5.3.1. The Over-expression construct

5.3.1.1. Generation of the mutant

In order to express RpaC off the *psbA2* promoter, primers *rpa*C-F and *rpa*C-R were designed which would flank the open reading frame sll1926.

The *Pst*1/*Sac*1 fragment excised from the PCR product was cloned into a compatibly digested pBluescript SK$^-$. A 2.1kb spectinomycin resistance cassette excised from pHP45ω was cloned into the *Hpa*1-linearised *rpa*C-containing pBluescript vector.

A pBluescript SK$^-$ plasmid containing the psbA2 promoter region and part of its coding sequence was made in a two step procedure using primers psbA2-F1, psbA2-R1, psbA2-F2 and psbA2-R2. First, the *Pst*1/*Sac*1 PCR product from psbA2-F1 and psbA2-R1 was cloned into pBluescript SK$^-$. The Nde1/Sac1 product from a PCR using psbA2-F2 and psbA2-R2 was cloned into the construct from the first step. The *Nde*1 fragment of the *rpa*C-spectinomycin resistance-containing construct was cloned into the *Nde*1-digested construct produced by the latter step.

The complete construct, shown in figures 5.1a and 5.1b was transformed into *Synechocystis* PCC 6803 wild-type and *Synechocystis* 6803 (glucose tolerant) *rpaC*$^-$ mutant cells. Transformants produced by homologous recombination were selected for on spectinomycin-containing BG11 medium (50 μg/ml) and spectinomycin and kanamycin containing medium (50 μg/ml) for the two respective mutants. Transformants were re-streaked repeatedly to encourage segregation.

Over-expression mutants do not express *psb*A2, and therefore, for control purposes, an insertional inactivation mutant of this gene was used for comparison, and grown on BG11 supplemented with 50 μg/ml kanamycin. A plasmid construct was donated by Dr. Peter Nixon, Imperial College London, transformed directly into *Synechocystis* 6803 and verified.

5.3.1.2. Genotypic characterisation

A PCR from the genomic DNA extracted from the *rpa*C^{-++} and *rpa*C^{++} mutants using primers 1926intF and aadAR yielded a fragment of approximately 250 bp. No product was obtained using aadAF with 1926intF, suggesting that the *aad*A cassette encoding the spec^R gene inserted in the same direction adjacent to the *rpa*C coding region. A psbA2-R2/aadAF primer combination yields a 1.2kb fragment, confirming that the rpaC-spec^R construct had indeed inserted into the *psb*A2 coding region. This is shown for *Synechocystis* PCC 6803 *rpa*C^{++} in figure 5.2. It can be concluded that neither over-expression mutant is fully segregated, as amplification of the wild type *psb*A2 gene in both cases suggests that there is a maximum copy number of *rpa*C which can be tolerated by the cells (data not shown).

Sequencing of the PCR product from the over-expression mutants confirms that *rpa*C inserted into the start site of *psb*A2 (data not shown).

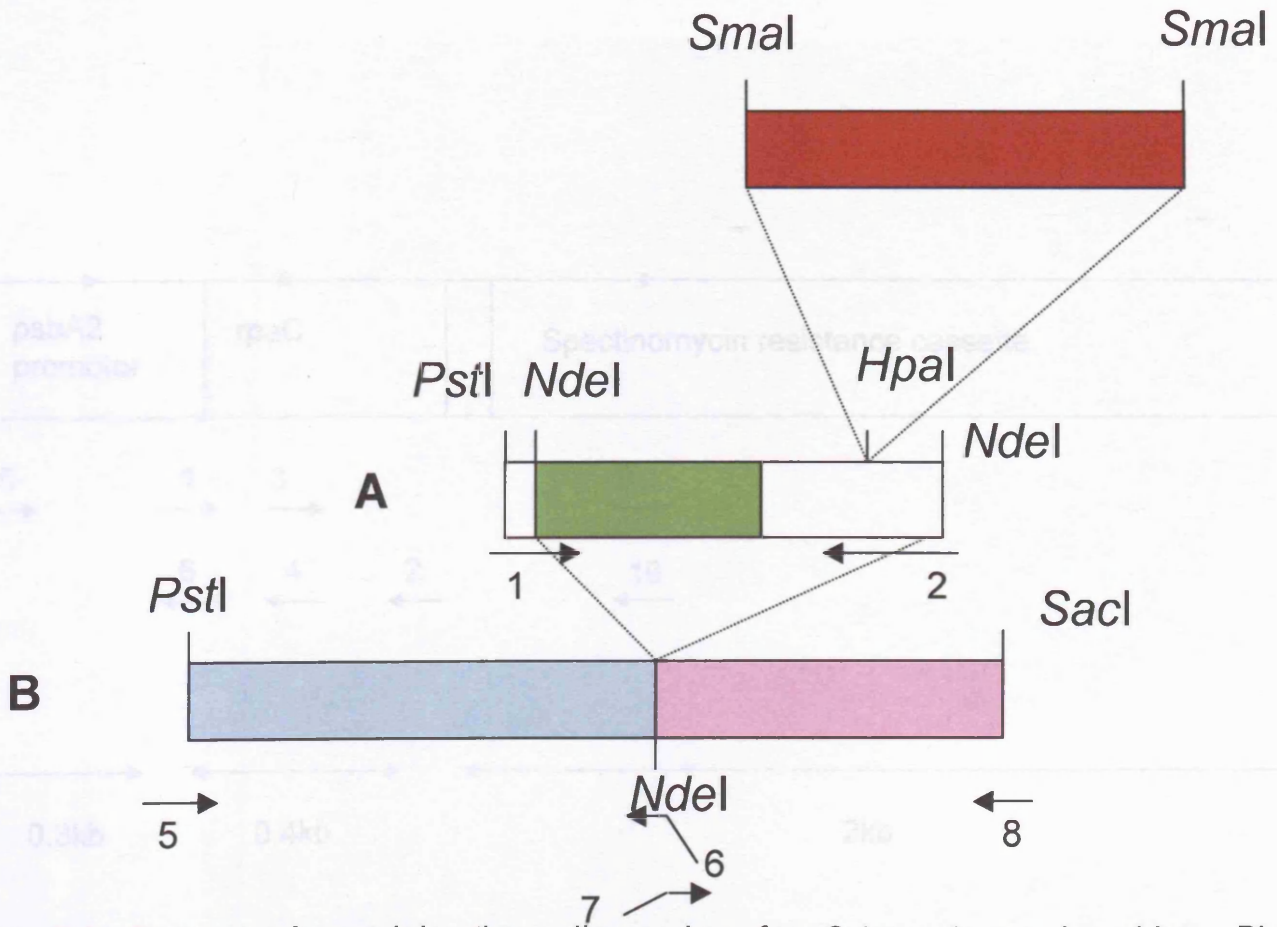


Figure 5.1a. Fragment A containing the coding region of *rpa*C (green) was cloned into pBluescript sk-, and the *Sma*I fragment containing the specinomycin resistance cassette (red) cloned into the *Hpa*Isite of A. Fragrment B was made in two cloning steps: first, the *Pst*I/*Sac*I fragment containing the *psb*A2 prmoter region (blue) was cloned into pBluescript. Separately, a *Pst*I/*Sac*I fragment containing PsbA2 coding region (lilac) was cloned into pBluescript. The *Nde*I/*Sac*I fragment excised from this latter construct was cloned into the *Nde*I/*Sac*I digested construct containing the promoter region, thus making B. The *Nde*I-digested A was cloned into the *Nde*I site within B, to give the complete construct. Relevant primers represented as numbered arrows (for details see table 2.1.)

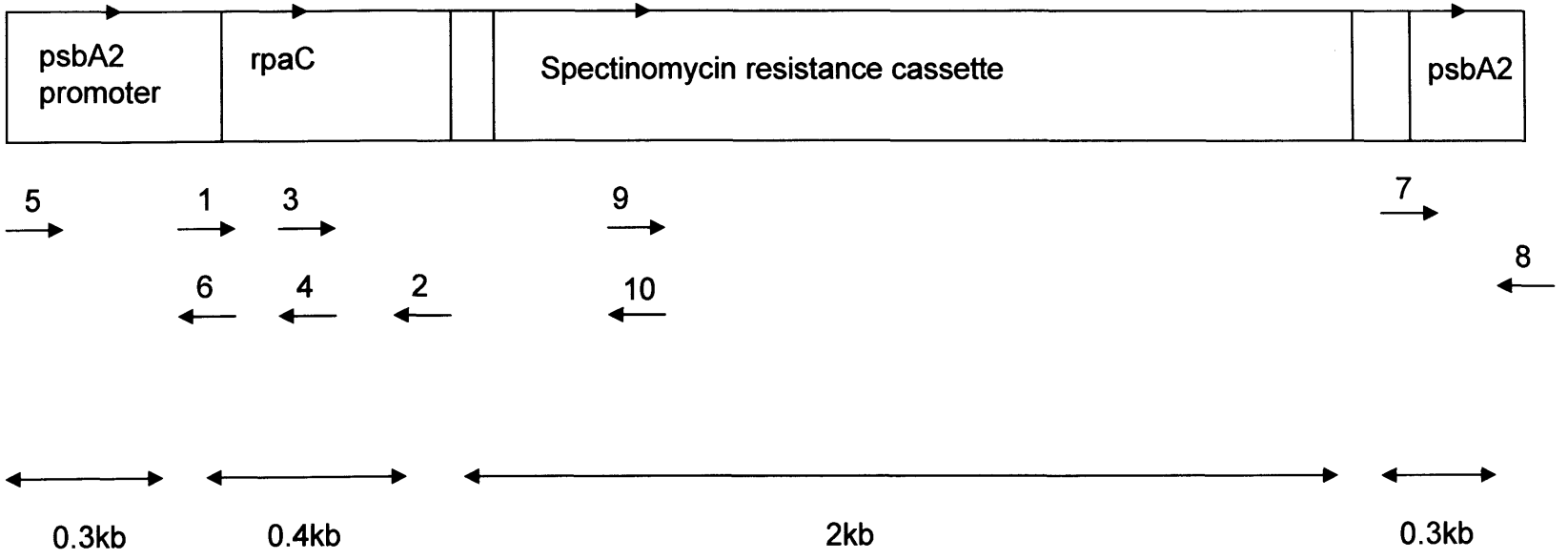


Figure 5.1b.Diagram showing the size of the construct used to over-express *rpa*C off the *psb*A2 promoter. Numbered arrows correspond to primers used to generate the construct or to genetically characterise (see chapter 2, table 2.1 for details).

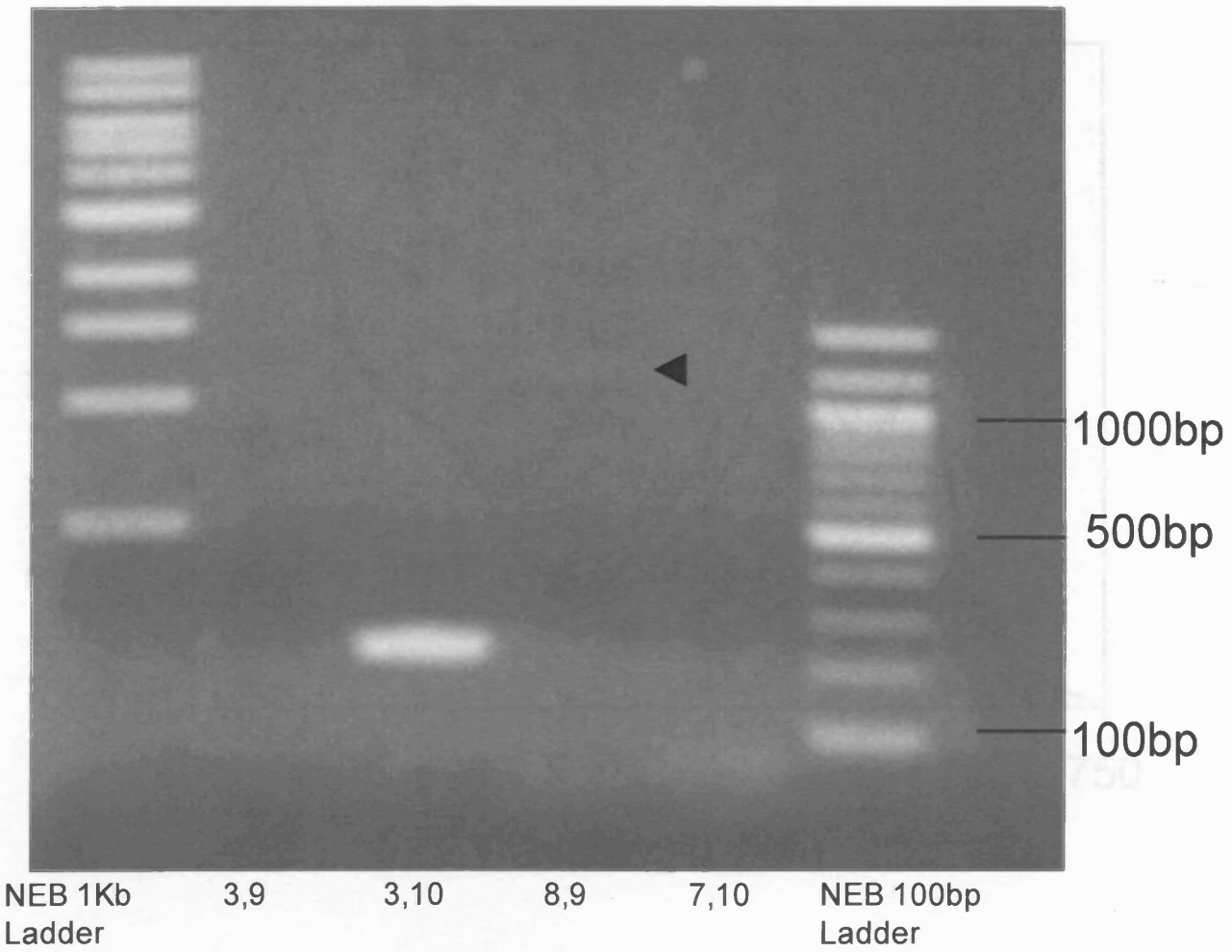


Figure 5.2. Gel photograph of PCR reactions confirming the correct sizes of the component parts of the over-expression construct in *Synechocystis* PCC. 6803. The faint band left of the ◄ denotes the 1.2kb PCR product. Primer combinations given at bottom of lane (see table 2.1 for details), size markers flanking.

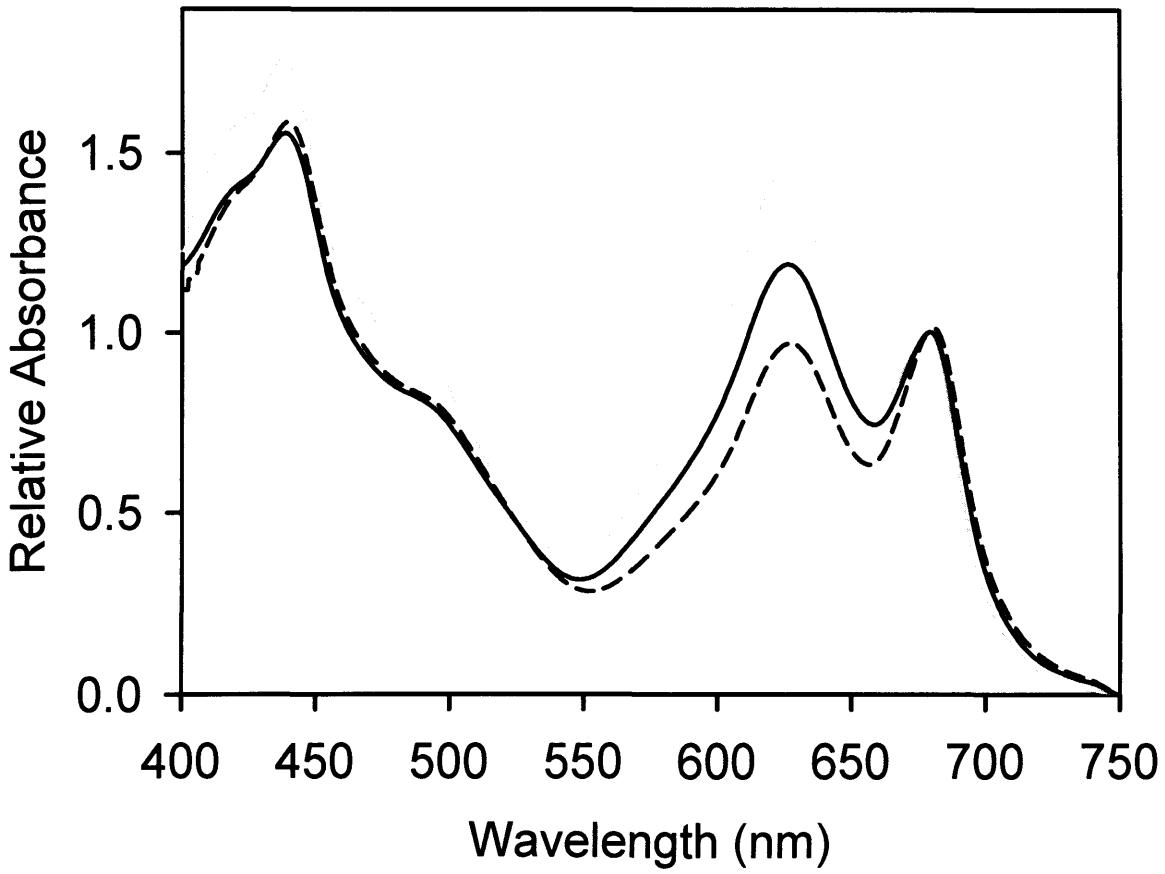


Figure 5.3. Absorption spectra for *S.* 6803GT *rpaC*$^{-}$ (black) and *psb*A2^{-} (dash) and *rpaC*$^{-++}$ (grey). Cells were normalised to the peak corresponding to chlorophyll a at 678nm.

	Synechocystis 6803 (GT)		
	*psb*A2$^-$	*rpa*C$^-$	*rpa*C^{-++}
Phycocyanin/cell	(2.7 ± 0.04) x 10^7	(2.4 ± 0.02)x10^7	(2.5 ± 0.3) x 10^7
Chlorophyll/cell	(6.8 ± 0.02) x 10^7	(6.3 ± 0.07)x10^7	(3.5 ± 0.03) x 10^7
Phycocyanin/Chlorophyll	0.32 ± 0.01	0.34 ± 0.04	0.71 ± 0.01
PSII/cell	134500 ± 2500	164300	224000 ± 1150
PSI/cell	521000	505000	233000
PSI/PSII	3.9	3.1	1.0

Table 5.1. Pigment content of the over-expressor (*rpa*C^{-++}) and the control strains (*psb*A2$^-$ and *rpa*C$^-$)

Synechocystis 6803 strain	Illumination conditions	Mean Doubling time (hours)	t-test (control vs *rpaC*$^{-++}$
psbA2^{-}	High	13±2	2.3, $p<8.6x10^{-2}$
rpaC^{-}	High	13±2	2.2, $p<8.9x10^{-2}$
rpaC^{-++}	High	24±8	
psbA2^{-}	Moderate	11.4±0.1	11.4, $p<3.3x10^{-4}$
rpaC^{-}	Moderate	12.0±0.04	12.8, $p<2.2x10^{-4}$
rpaC^{-++}	Moderate	23±2	
psbA2^{-}	Low	34±2	8.54, $p<0.013$
rpaC^{-}	Low	35±1	12.38, $p<0.0065$
rpaC^{-++}	Low	49±1	
psbA2^{-}	Blue	72±4	$p<0.23$
rpaC^{-}	Blue	75±5	$p<0.18$
rpaC^{-++}	Blue	72±1	

Table 5.2. Summary of mean doubling times (with standard deviations) of mutant and control strains (n = 3 in all cases) under different illumination conditions.

5.3.2.4. Oxygen evolution

Figure 5.4 shows the rate of oxygen evolution for the test and control strains which had been grown under moderate illumination conditions. Oxygen evolution may be used to determine the total activity of PSII. The rate of oxygen evolution per chlorophyll in the over-expression mutants is significantly higher than in the control strains, $p<0.002$ and 0.03 for t-tests comparing the over-expressor *rpa*C^{-++} with *rpa*C^{-} and *psb*$A2^{-}$ respectively). The saturating light intensities for the *rpa*C^{++} and *rpa*C^{-++} strains are lower than for the controls, as well as the maximal rates being higher in the test strains. This may suggest that light harvesting to PSII is increased as a result of there being more RpaC than is usual. Alternatively, the result may be attributed to greater quantities of PSII or smaller amounts of PSI in the thylakoids.

5.3.2.5. State transitions

Wild type *Synechocystis* 6803 is capable of state transitions, while an *rpa*C inactivation mutant of *Synechocystis* 6803 is not (Emlyn-Jones *et al*, 1999). An inactivation mutant in the non-glucose tolerant strain of *Synechocystis* 6803 was made (chapter 4 of this thesis) and exhibits a similar phenotype. Whole cell fluorescence emission spectra were obtained at 77K to determine the state transition competence of the RpaC over-expressors, *Synechocystis* PCC 6803 *rpa*C^{++} and *Synechocystis* 6803 *rpa*C^{-++}. Cells were frozen after 5 minutes of red light or the same time in the dark, and by exciting at 600 nm, a measure of energy transfer to the reaction centres from the phycobilisomes may be obtained. Figure 5.5 depicts such spectra from cells grown in different illumination regimes. The data show that *psb*$A2^{-}$ cells are capable of state transitions, particularly under dim illumination, but that $RpaC^{-}$ cells are not, regardless of the growth conditions (as previously reported by Emlyn-Jones *et al*, 1999, and Emlyn-Jones, PhD thesis, 2000). Over-expression of RpaC on an *rpa*C^{-} background (that is, the mutant designated *rpa*C^{-++}) does not rescue a state transition phenotype. The over-expression mutant gives a ratio of fluorescence at 654nm/680nm a little lower in the test strain than in the control strains, perhaps suggesting the mutant is more state 1-like. This difference is also seen in *Synechocystis* PCC 6803 *rpa*C^{++}.

The chlorophyll effect on state transitions is seen when exciting the frozen samples at 435nm. It is only seen in figure 5.6 in the *psb*$A2^{-}$ mutant grown under low

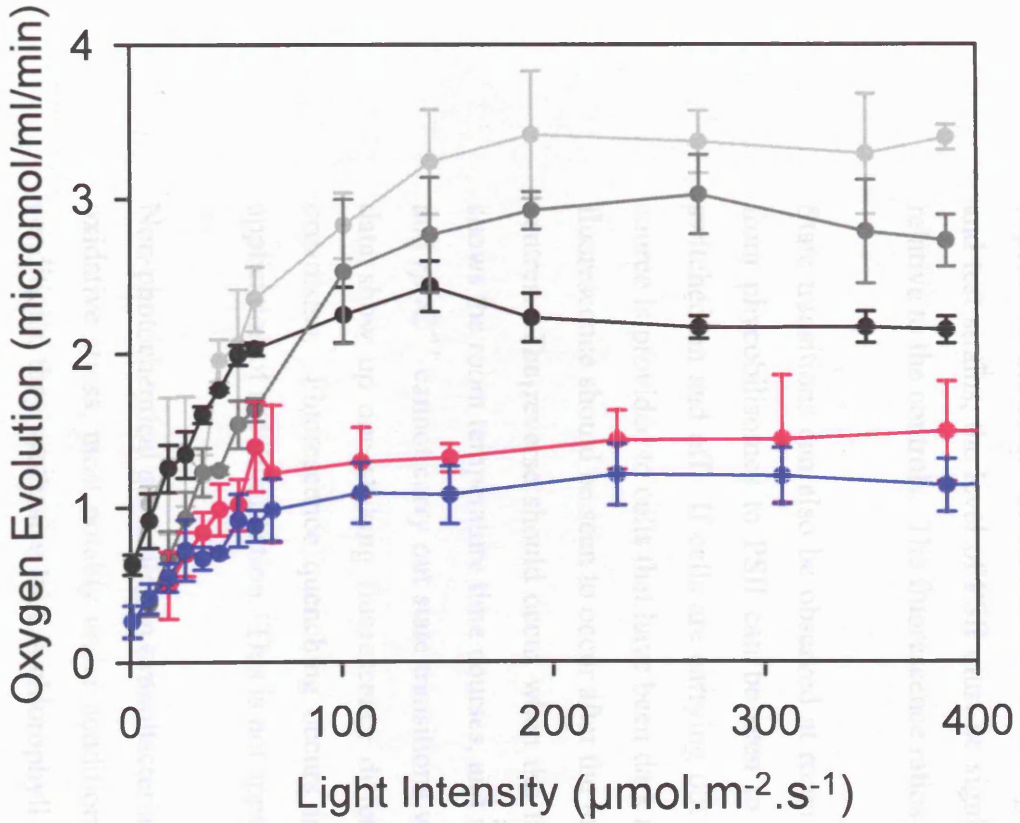


Figure 5.4. Oxygen evolution of wild type *S.* PCC 6803rpaC- (black), *S.* GT 6803*rpaC*$^{-}$ (blue), *S.* GT 6803*psb*A2^{-} (pink), *S.* PCC 6803*rpaC*$^{++}$ (grey) and *S.* GT 6803*rpaC*$^{-++}$ (dark grey) using red wavelength illumination under increasing light intensities. Cells had been cultured under mid illumination conditions. n=3, with standard deviations given.

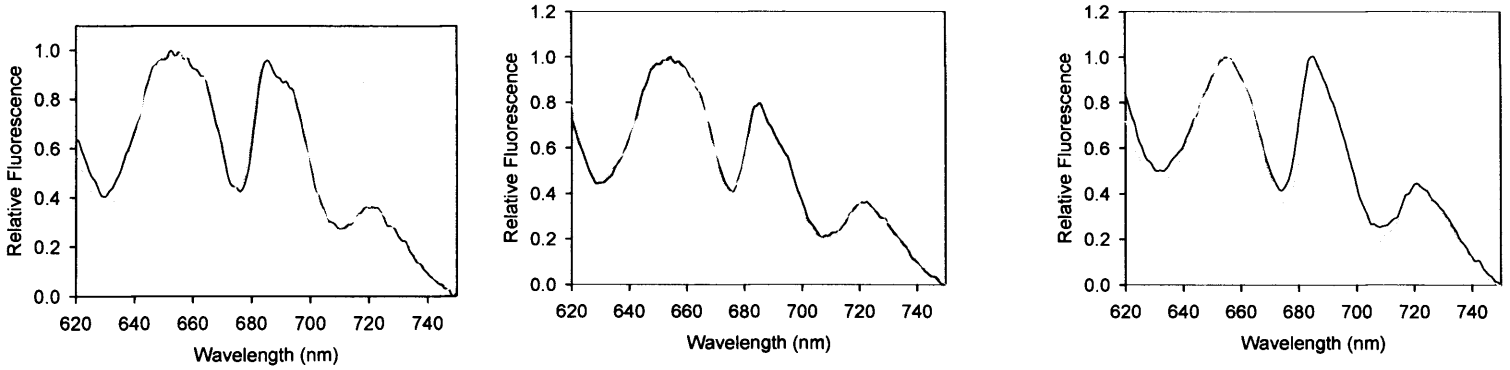
illumination conditions, where the phycobilisome effect is also evident (figure 5.5). The *rpa*C^{-++} mutant does not give higher PSII/PSI fluorescence after being provided with red light compared with cells incubated for 5 minutes in the dark, suggesting that even the "state transition" some attribute to "spill-over" is not occurring either. This may be due to excess RpaC cluttering the already crowded membrane. The spectra shown in figure 5.6 also suggest that the PSII/PSI fluorescence ratio is far larger in the test strain than in the control organisms, 0.6 approximately, compared with 0.4. This supports the observed up-regulation of oxygen evolution in the over-expression mutants. Given that PSI is not significantly different between controls and test strains, the level of PSII must be significantly increased in the test strains relative to the controls. The fluorescence ratios hint at this being the case.

State transitions can also be observed at room temperature, where energy transfer from phycobilisomes to PSII can be seen to be altered as a red light source is switched on and off. If cells are carrying out state transitions, when the red light source is provided to cells that have been dark adapted for 5 minutes, a slow rise in fluorescence should be seen to occur after the initial drop caused by opening of PSII centres. The reverse should occur when the light source is removed. Figure 5.7 shows the room temperature time courses, and reinforces the observation that *rpa*C^{-} and *rpa*C^{-++} cannot carry out state transitions while the *psb*A2^{-} mutant can. These data show up one striking fluorescence difference between the test and control organisms. Fluorescence quenching occurs in the over-expression mutant upon application of red illumination. This is not apparent in the control strains.

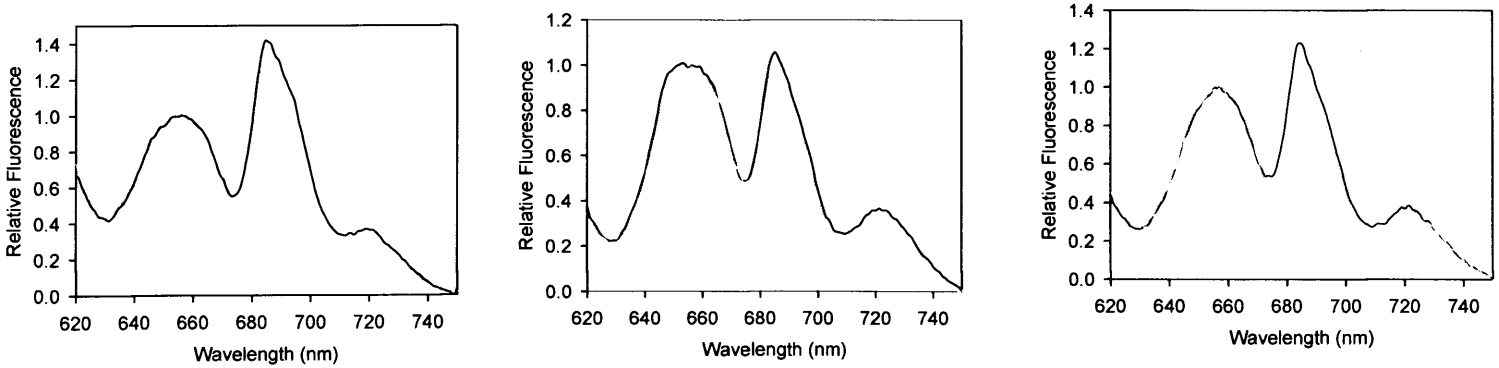
Non-photochemical quenching in cyanobacteria has been observed in cells suffering oxidative stress, most notably under conditions of iron deprivation. Under these conditions, they synthesise IsiA, a chlorophyll molecule which can complex to aid light harvesting to PSI, but can also prevent excess light energy damaging the reaction centres.

The presence of IsiA is suggested by a large peak at 680nm in a 77K fluorescence emission spectrum showing chlorophyll excitation (Burnap *et al*, 1993). This peak is not obviously apparent in the spectra shown in figure 5.6, though there may be a hint of IsiA in the test strains grown under high intensity white light (confirming a

1a



2a



3a

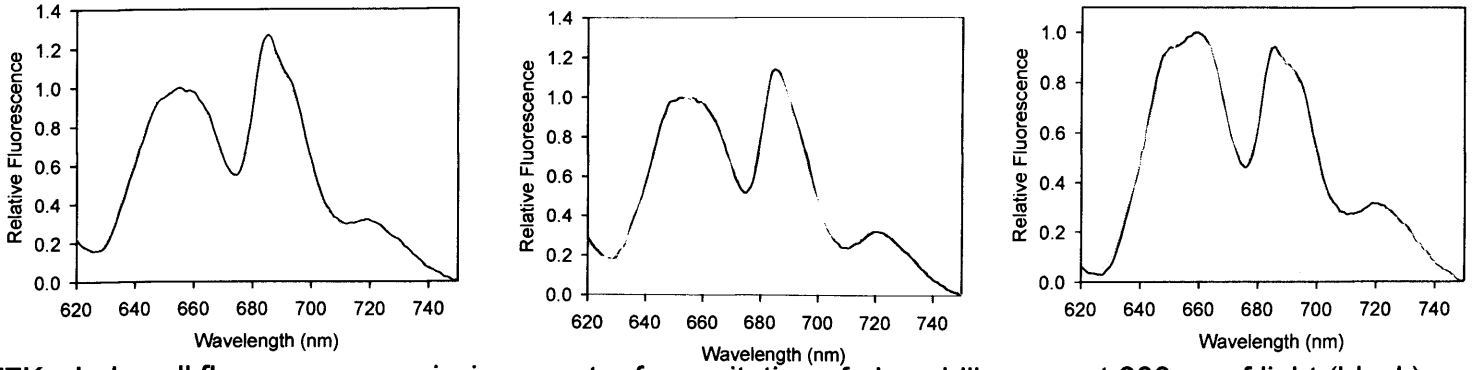


Figure 5.5. 77K whole cell fluorescence emission spectra for excitation of phycobilisomes at 600nm of light (black) or dark (grey) adapted *Synechocystis* 6803 GT*psb*A2^{-}, *rpa*C^{-} and *rpa*C^{-++} (1-3 respectively) under high, mid and low illumination growth conditions (a, b and c respectively). Spectra were obtained using excitation and emission slit widths of 5nm, and were normalised to phycocyanin fluorescence at 650nm.

1a

2a

3a

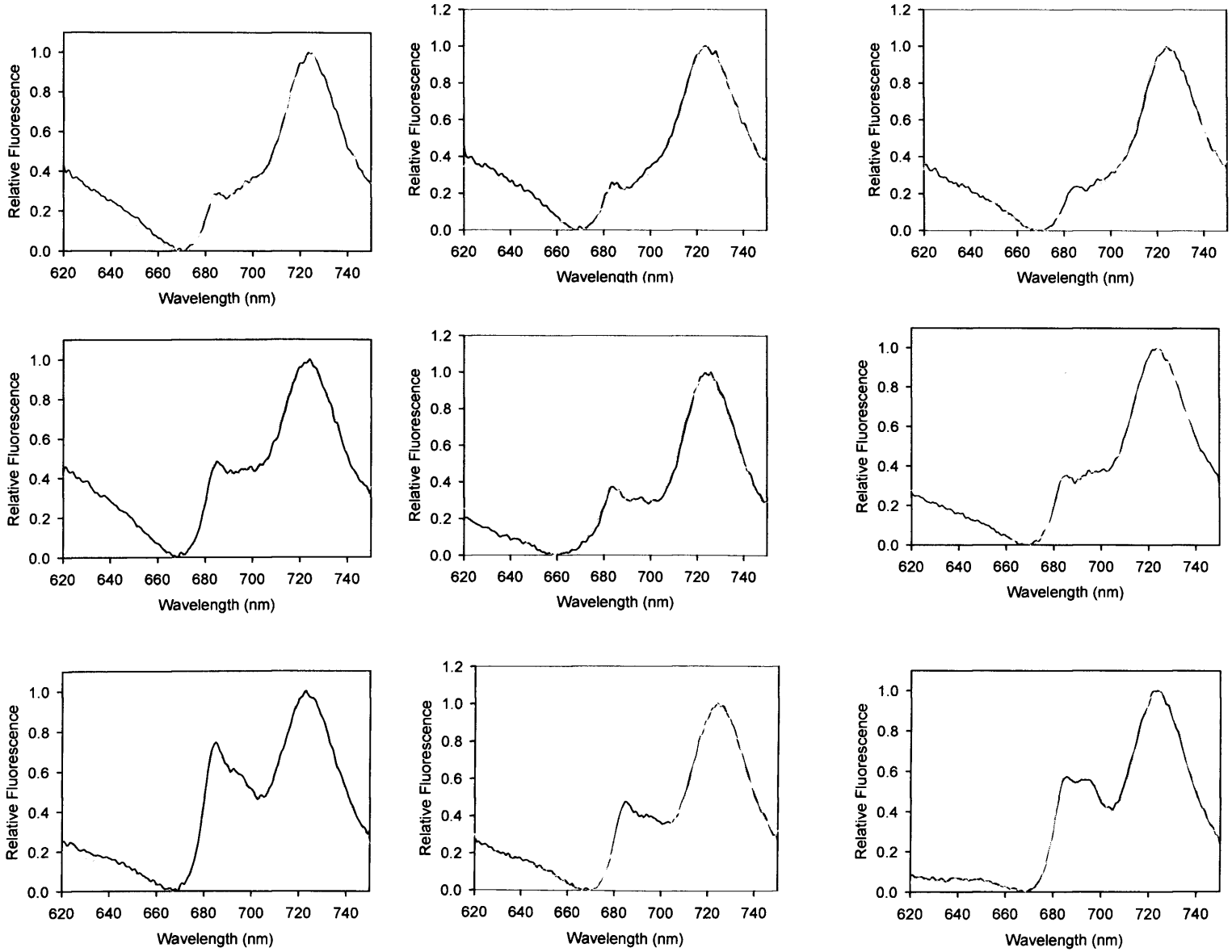


Figure 5.6. 77K whole cell fluorescence emission spectra for excitation of chlorophyll at 435nm of light (black) or grey (dark) adapted *S.* 6803 GT*psb*A2^{-}, *rpa*C^{-} and *rpa*C^{-++} (1-3 respectively) under high, mid and low illumination growth conditions (a, b and c respectively). Spectra were obtained using excitation and emission slit widths of 5nm, and were normalised to chlorophyll a fluorescence at 720nm.

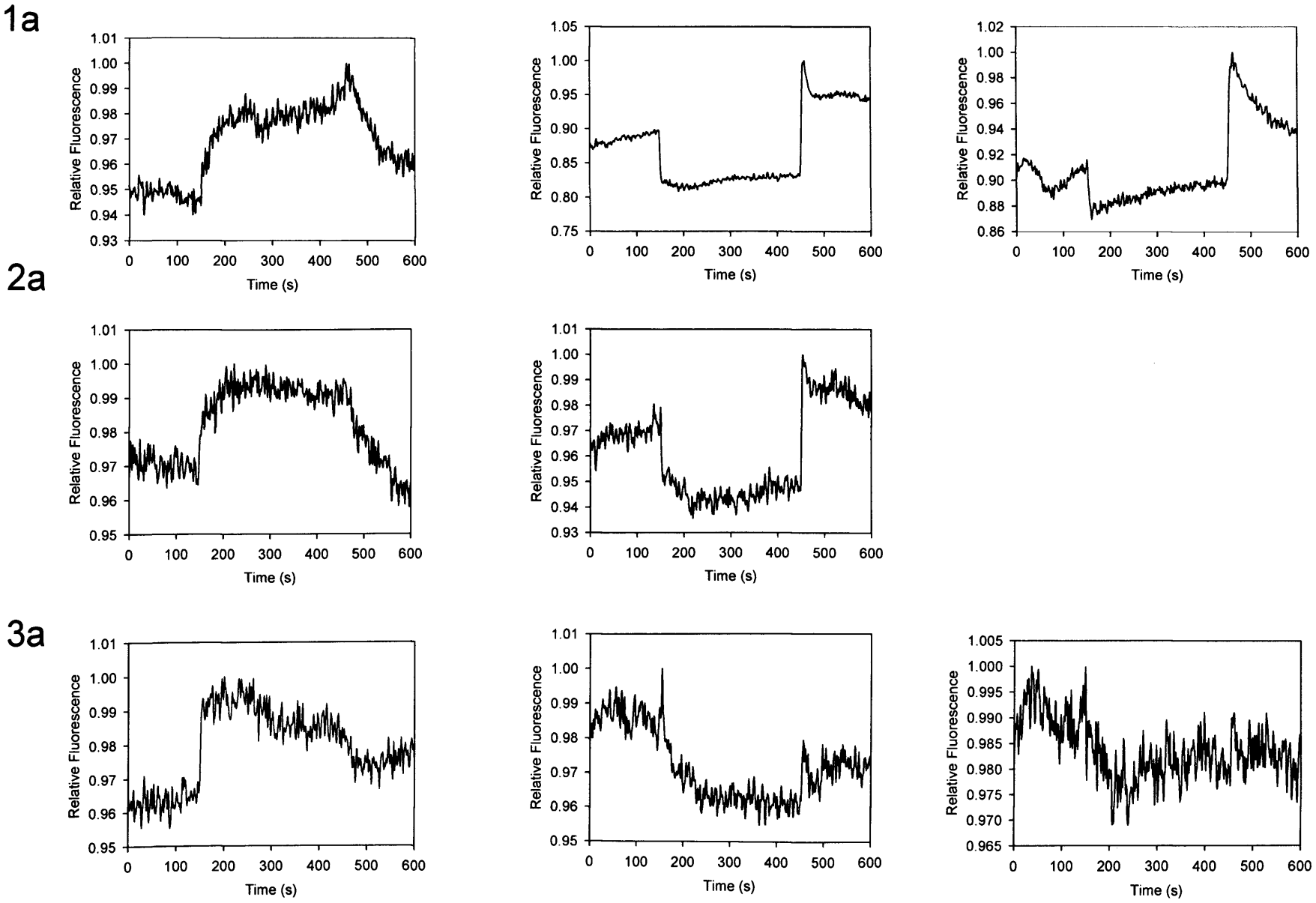


Figure 5.7. Room temperature timecourse, for excitation of phycobilisomes at 600nm and emission at 680nm, corresponding to PSII. Traces presented are for *S.* 6803 GT*psb*A2^{-}, *rpa*C^{-} and *rpa*C^{-++} under high, mid and low illumination growth conditions (a, b and c respectively). Spectra were obtained using cells concentrated to 5μM chlorophyll, with excitation and emission slit widths at 10nm and 5nm. Red illumination was provided between 150s and 450s, otherwise cells received dim yellow illumination.

greater degree of oxidative stress). Since quenching of fluorescence was observed in cultures grown under a range of illumination conditions, it is unlikely that the quenching effect observed in the over-expressor is attributable to large quantities of IsiA. Further, IsiA-dependent quenching seems to be specifically triggered by blue light (Cadoret *et al*, 2004), and the quenching observed here was induced by red wavelength illumination.

The over-expression mutant in the non-glucose tolerant strain of *Synechocystis* 6803, dubbed *Synechocystis* 6803 *rpa*C^{++} may be less segregated than the glucose-tolerant version. It gives a positive state transition phenotype (spectra given in figure 5.8), and this may be because it retains a wild type gene (which may affect where it is targeted to, if a particular pre-sequence was necessary for appropriately timed and targeted expression). An alternative explanation may be that less of the protein is made, resulting in a less extreme phenotype. This could be achieved simply by selecting for secondary mutations within the over-expression construct which have generated stop codons within the coding sequence, yielding truncated proteins which may be less harmful to the mutants. *Synechocystis* PCC 6803 *rpa*C^{++} also grows slower than the control strain, exhibits a larger PSII/PSI ratio, evolves oxygen at an increased rate and possesses pigment quantities more similar to the *Synechocystis* 6803 *rpa*C^{-++} mutant than the control strains.

5.3.2.6. Phycobilisome diffusion

FRAP experiments were carried out on mutant and control strains as described in section 2.12, with appropriate modifications as described in section 7.4.3. Phycobilisomes diffuse completely in the mutants over 30 s time-scales, suggesting that upregulating the transcription of *rpa*C mRNA does not inhibit the mobility of the light harvesting complexes.

5.3.2.7. PSII diffusion

FRAP experiments were undertaken as described earlier. PSII is apparently immobile in all strains, as expected. That chlorophyll diffusion is not observed is further confirmation that IsiA is not present, as it has been shown previously, in *Synechococcus* 7942 (Sarcina and Mullineaux, 2004), that IsiA complexes are capable of slow diffusion when expressed in iron stressed cells.

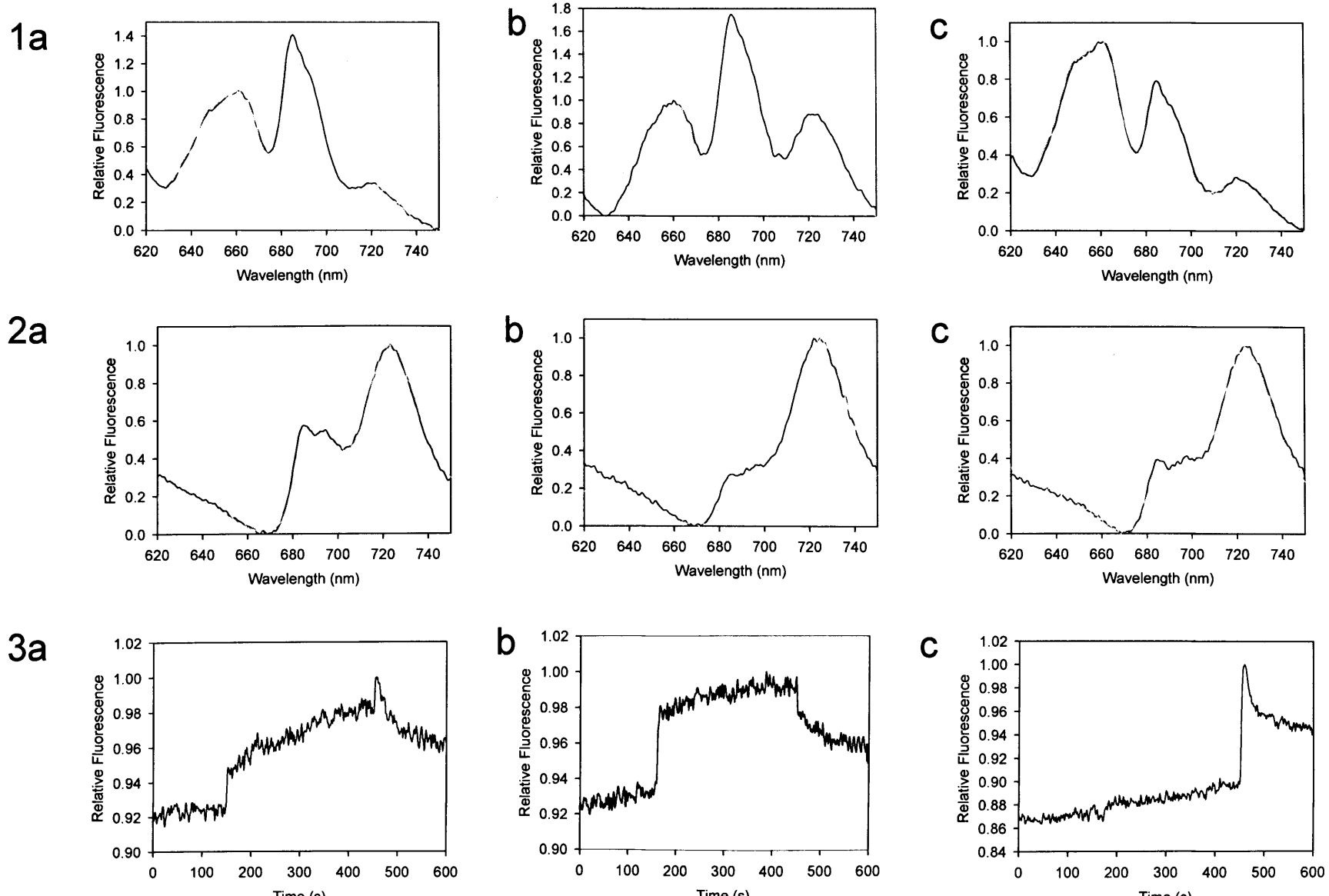


Figure 5.8. 77K whole cell fluorescence emission spectra for excitation of phycobilisomes at 600nm (1) and chlorophyll at 435nm (2) of light (black) or dark (grey) adapted *S.* PCC 6803 *rpa*C^{++} grown under high, mid and low illumination growth conditions (a, b and c respectively). Spectra were obtained using excitation and emission slit widths of 5nm, and were normalised to phycocyanin fluorescence at 650nm. 3a-c depicts the corresponding room temperature time courses as previously described.

5.4. Discussion

The gene encoding RpaC was cloned into the start site of *psb*A2, a constitutively expressed gene in *Synechocystis* 6803. Pleiotropic effects were observed as a result of this one mutational event.

The mutant displays high-light sensitivity and exhibits non-photochemical quenching (NPQ) of fluorescence. NPQ has a protective role, in that it prevents photo-damage to the reaction centres (particularly PSII) under conditions of excess illumination when cells are under oxidative stress. On a simplistic level, this could mean that an excess of RpaC in the thylakoid membrane results in more energy transfer to the reaction centres, probably PSII. It could be postulated that RpaC could increase the adherence of the phycobilisomes to PSII at the expense of PSI. This hypothesis would be consistent with findings in *Synechococcus* 7942 *rpa*C$^-$ (chapter 4), where a reduction in copy number of the *rpa*C gene resulted in weaker affinity of the phycobilisomes for PSII. A PSI$^-$ mutant of *Synechocystis* 6803 *rpa*C$^-$ (Emlyn Jones, PhD thesis, 1999) showed a notable decrease in energy transfer from phycobilisomes to PSII as compared with the single *rpa*C$^-$ mutant, also lending weight to this theory. There was no corresponding difference in phycobilisome to PSI energy transfer in a double mutant of *Synechocystis* 6803 *rpa*C$^-$ in a PSII-background (Emlyn-Jones, PhD thesis, 1999)

It is somewhat difficult to determine whether this hypothesis holds true, as cells respond to excess energy transfer (which presumably causes photodamage) by decoupling phycobilisomes from the reaction centres. This is observed in 77K fluorescence emission spectra, where the peak at 680nm, corresponding to the terminal emitters of the phycobilisomes is seen to be elevated following a 5 minute period of red light illumination. The ratio of fluorescence of PSII/PSI from phycobilisomes does not appear to be different under state 1 or state 2 illumination conditions, suggesting that the *Synechocystis* 6803 *rpa*C^{-++} cells are not carrying out state transitions (figure 5.5). This does not appear to be the case for *Synechocystis* 6803 *rpa*C^{++}, a finding confirmed using a room temperature time-course measurement where fluorescence of the phycobilisomes binding to PSII is seen to rise upon illumination (state 1) and fall characteristically when the light is removed

(state 2) (figure 5.8). A number of different explanations for these features are possible.

First, it is possible that the mutant in the non-glucose tolerant strain remains state transition competent on account of it retaining a wild-type copy of *rpa*C. It is unlikely, though still possible, that the over-expression mutants do not effectively target *rpa*C mRNA/protein to the correct location on account of *rpa*C having been moved to another promoter. This would only really be feasible if there is a pre-sequence missing in the over-expressor. Might transcription of *rpa*C be regulated by the expression of an unknown compound which targets the wild type promoter for wild type *rpa*C when appropriate? It is possible, but no information about the signalling pathway renders the ability to test this hypothesis impossible. *rpa*C is not located within an operon in *Synechocystis* 6803, and its neighbouring genes have both been previously knocked out (Emlyn-Jones *et al*, 1999) and shown no phenotypic characteristics different from wild type cells.

An increased affinity for PSII reaction centres for phycobilisomes may be detrimental to the cells under most conditions owing to it promoting damage to D1 complexes. This might explain why *Synechocystis* 6803 only usually expresses *rpa*C at low levels. However, when cells are exposed to higher doses, it is not unreasonable to suggest that the cells could respond as a result of receiving signals about the redox status of intersystem electron carriers and attempt to subvert the danger of excess energy transfer by decoupling the phycobilisomes.

Another possible cause for the decoupling of the phycobilisomes might be that the excess of RpaC in the thylakoid membrane actually gets in the way of efficient association to PSII. If RpaC was both the signal to switch from state 2 conformation to a state 1 type as well as being a PSII binding factor increasing the affinity of the phycobilisomes for it, this could also be a possible reason. Alternatively, if the normally PSII-associated RpaC, accumulates free in the membrane when in excess, upon transition to state 1, the phycobilisomes could bind to RpaC. This could account for the observed quenching of fluorescence triggered by red light.

It will be a difficult task to delineate all the factors which contribute to the multiple adverse phenotypes exhibited by the *rpa*C^{-++} and *rpa*C^{++} mutants. Whilst there may be an abundance of RpaC in the membranes of these mutants, it is unreasonable to use them as a basis for generating GFP-RpaC off the *psb*A2 promoter. This is simply because, on the evidence of the data presented here, it cannot be determined whether *rpa*C at this location in the cyanobacterial genome is producing a loss of function or a gain of function phenotype.

Chapter 6: Phycobilisome diffusion and IsiA-induced Non-Photochemical Quenching

Chapter 6: Phycobilisome diffusion and IsiA-induced Non-Photochemical Quenching

6.1. Objective

- To determine if phycobilisome mobility is required for non-photochemical quenching of fluorescence in iron-starved *Synechocystis* 6803

6.2. Introduction

6.2.1. IsiA

All living organisms require iron. Photoautotrophic organisms are no exception, many of the components of the photosynthetic reaction centre and electron transport chain themselves containing iron. *Synechococcus* sp. PCC 7942 and *Prochlorococcus* species are commonly found in iron-limited habitats. It is therefore not surprising that they have evolved mechanisms of scavenging iron, but also reliable means to protect their photosynthetic reaction centres (in particular, PSII) from oxidative damage brought about by the formation of oxygen free radicals prompted to form when excitation energy is not efficiently trapped by iron-depleted cells. Further, when iron is limited, enzymes such as superoxide dismutase (SOD) which decompose the radicals are also in short supply as they require iron either as an intrinsic component or as a cofactor.

Under conditions of extreme iron deprivation, a 37kDa protein in possession of six transmembrane helices termed IsiA is expressed in cyanobacteria (Burnap *et al*, 1993). IsiA displays strong homology to proteins encoded by the *pcb* genes of *Prochlorococcus,* and the cyanobacterial *psb*C gene known to encode the intrinsic core antenna CP43 protein of PSII. It is therefore also commonly known as CP43' or IsiA. Its chlorophyll binding properties are conserved (Bricker and Frankel, 2002). A key difference between the two proteins is that IsiA does not contain the sequence of approximately 100 amino acids of a hydrophobic loop on the luminal side of the protein, between helices V and VI (Burnap *et al*, 1993). The respective roles of CP43 and IsiA are distinct, as it has been shown in *Synechocystis* 6803 that IsiA is unable to compensate for the absence of CP43 even when the expression of the former is induced by iron stress (Rogner *et al*, 1991).

The function of IsiA has been under consideration for many years. It is thought to play two key roles, in photoprotection of the reaction centres (PSII in particular, which is more significantly prone to oxidative damage) (Park *et al*, 1999; Sandstrom *et al*, 2001) and as a light harvesting complex under iron limited conditions (Pakrasi *et al*, 1985). The latter has been confirmed in both *Synechococcus* sp. PCC 7942 and *Synechocystis* sp. PCC 6803 (Boekema *et al*, 2001 and Bibby *et al*, 2001a, b respectively), where under iron stress, a closed ring of 18 IsiA molecules forms around PSI trimers to make a PSI-supercomplex, the ring acting as a chlorophyll antenna, functionally transferring light energy to the reaction centre (Melkozernov *et al*, 2003; Andrizhiyevskaya *et al*, 2004). This antenna ring appears similar to the light harvesting Pcb proteins found in *Prochlorococcus* species (Bibby *et al*, 2001c). That PSI requires assistance under iron stress is not surprising, as the three iron-sulphur clusters within it provide ample reason for why PSI is so scarce when iron is in short supply (Falk *et al*, 1995).

Until recently, it has been rather more difficult do demonstrate the photoprotective role of IsiA, particularly with respect to its role in shielding PSII. However, it has been recorded that IsiA is expressed in excess of what is required for efficient light harvesting to PSI, and further, that supercomplexes of IsiA can be found in the absence of PSI (Yeremenko *et al*, 2004). This suggests that it may have a role in photoprotection.

It has been reported that where iron is scarce, the cyanobacterial light harvesting complexes, the phycobilisomes, are produced sparingly, and light harvesting by phycobiliproteins to PSII has been shown to be reduced (Sandstrom *et al*, 2002). It would be of great interest to determine whether IsiA mobility and/or phycobilisome diffusion is a requirement for quenching of excitation energy in stressed *Synechococcus* 7942, an organism which we can efficiently measure diffusion rates of the strongly fluorescent chromophore containing molecules.

6.2.2. Non-Photochemical Quenching (NPQ)

Photosynthetic organisms are capable of regulating their light harvesting apparatus in response to their light environment. In bright light, cells are provided with

saturating light intensities in excess of their photosynthetic requirements. In plants, a well-characterised phenomenon known as Q_E (a form of Non-Photochemical Quenching (NPQ)) is induced under these conditions (see Horton *et al*, 1996 and Holt, *et al*, 2004, for reviews). Here, the excess excitation is dissipated as heat, presumably to prevent photodamage to the reaction centres. When NPQ is induced, a decrease in fluorescence from chlorophyll *a* may be observed. The quenching mechanism in eukaryotes is known to involve carotenoids, specifically the de-epoxidation of violaxanthin to zeaxanthin in the light harvesting antennae (Demming *et al*, 1987; Demming, 1990; Gilmore *et al*, 1991). The PSII subunit dubbed PsbS is also thought to be directly involved in Q_E induction (Li *et al*, 2000) in plants.

NPQ has recently been observed in cyanobacterial cells deprived of iron, specifically under conditions where IsiA is strongly expressed. The mechanism of quenching in cyanobacteria is likely to be distinct from that of Q_E in plants, as the prokaryotes do not possess an accessory chlorophyll-type antenna for light harvesting. It has, however, been reported that cyanobacteria have a *psb*S-like gene, similar to that found in spinach, but little is known about its product (Wedel *et al*, 1992, for example).

Following iron deprivation, NPQ of variable fluorescence (F_v) and minimal fluorescence (F_0) is observable in cyanobacteria, but the latter occurs to a greater extent after a prolonged period of starvation (Bailey *et al*, 2005). Fluorescence quenching occurs over a few minutes during exposure to bright light and is reversible upon removal of the illumination source (Cadoret *et al*, 2004; Bailey *et al*, 2005).

Quenching appears to be wavelength dependent, occurring when cells are exposed to blue light (Cadoret *et al*, 2004; Rakhimberdieva *et al*, 2004). Unlike in plants where induction of NPQ results from the acidification of the thylakoid lumen, the mechanism in cyanobacteria does not appear to be pH-dependent, where NPQ is unaffected by the presence or absence of un-couplers (Bailey *et al*, 2005).

6.3. Rationale

In chapter 3, it was demonstrated that phycobilisome mobility was required for the occurrence of state transitions, a rapid light-adaptation mechanism involving the re-distribution of excitation energy from one reaction centre to another depending on the illumination conditions cells were provided with. This observation was made by arresting cells in state 1 or state 2 using high osmotic strength buffers. It was shown that cells fixed in this way, with a larger proportion of phycobilisomes fixed in their association with one reaction centre or the other were unable to diffuse across the thylakoid membranes. This is probably owing to an increased binding stability of the phycobilisomes to the reaction centre complexes.

It has been shown that IsiA complexes in the thylakoid membrane are mobile with diffusion coefficients of about 3 x $10^{-11}cm^2s^{-1}$ (Sarcina and Mullineaux, 2004). It is of interest to determine if phycobilisome diffusion is necessary for NPQ.

6.4. Results

6.4.1. NPQ in the presence of phosphate

Synechocystis 6803 was cultured by Shaun Bailey (Warwick University) for 20 days in the absence of iron components in growth medium results in the induction of IsiA expression. (This is observable in fluorescence emission spectra for chlorophyll excitation given by figure 6.2A and 6.3A, and is discussed later). This protein is believed to aid light harvesting to the photosynthetic reaction centres under conditions of iron starvation (Bibby *et al*, 2001). It has been previously shown that IsiA is produced in excess of that required for light harvesting, and that IsiA forms complexes with itself in the thylakoid membrane (Yeremenko *et al*, 2004). This has led to the suggestion that these complexes serve to prevent photoinhibition (particularly of PSII) under oxidative stress caused by iron starvation. It has been proposed that this is achieved in some way by non-photochemical quenching of fluorescence. Using moderate actinic irradiance, stressed cells quench F_0, and fluorescence recovery is complete after incubation for 1 hour in the dark post illumination (Bailey *et al*, 2005).

Figure 6.1 presents the PAM fluorescence traces of *Synechocystis* 6803 with and without 1M phosphate buffers. The traces shown are for 20-day iron starved cells.

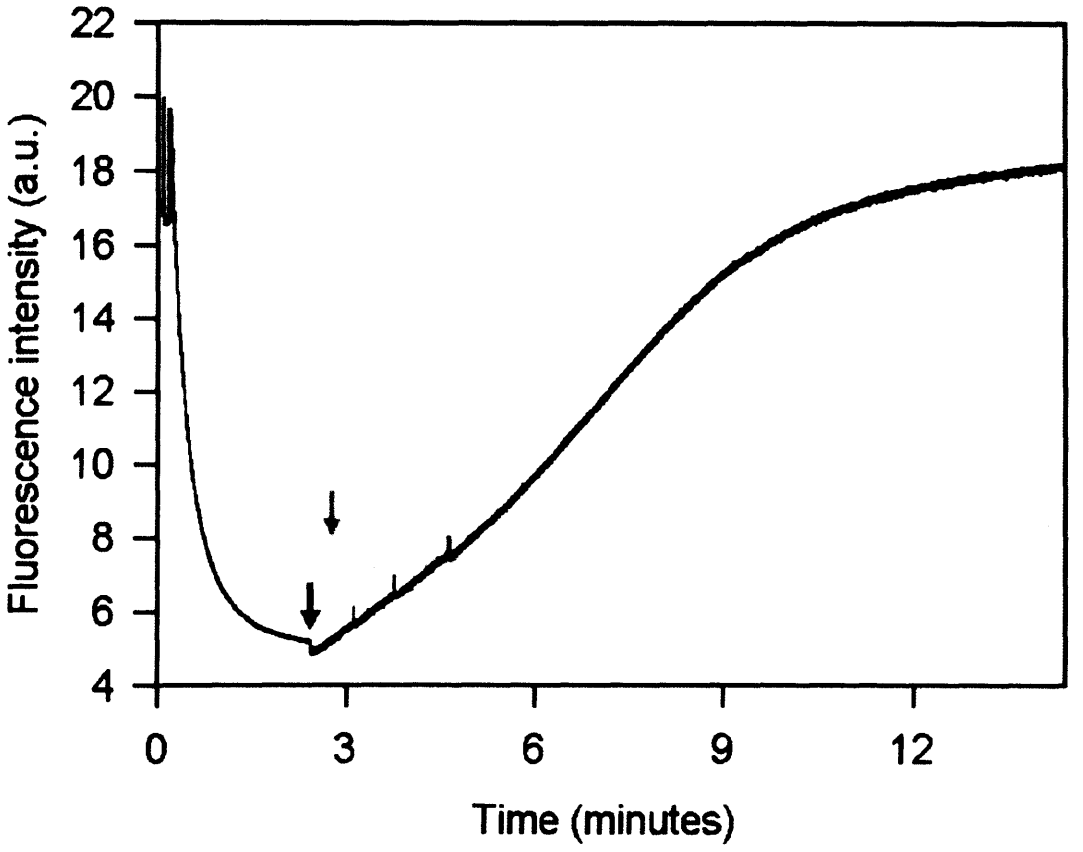


Figure 6.1. PAM fluorescence traces of iron deprived *Synechocystis* 6803 with (grey) and without (black) 1M phosphate buffer. Cells are in the light at time 0. The arrow indicates the time when illumination was removed. Small spikes in the traces were achieved by applying small pulses of high intensity illumination, and show the amount of photochemical quenching present.

As demonstrated previously (Bailey *et al*, 2005) the data demonstrate that iron stressed cells show NPQ-type quenching when given excess white light, while non-stressed bacteria exhibit a transition to state 1 in the same conditions (not shown). When the light source is removed, fluorescence from stressed cells recovers.

It has been demonstrated previously that treatment of cyanobacterial cells with high osmotic strength buffers is able to arrest cells in light state 1 or 2 by inhibiting the movement of the light harvesting complexes, the phycobilisomes (Joshua and Mullineaux, 2004). This observation may be interpreted as the buffers increasing the affinity of the phycobilisomes with the reaction centres they were associated with upon their addition. The black line in figure 6.1 depicts a fluorescence trace which shows that incubating iron-starved cells in 1 M phosphate buffer (pH 6.8) prior to light acclimation does not inhibit the non-photochemical quenching. However, very little recovery upon subsequent incubation in the darkness is observed in phosphate-treated cells (grey). This phosphate treatment has no effect on the quenching of F_v (Shaun Bailey, Warwick University, personal communication) which is seen to occur during the early stages of iron deprivation (data not shown).

6.4.2. Phycobilisomes and energy transfers to reaction centres and IsiA?

In order to demonstrate that phycobilisomes were present in iron stressed as well as non-stressed cells, absorbance spectra were recorded between 450 nm and 750 nm (Shaun Bailey, Warwick University, Personal communication). They confirm that the phycobilisomes are not lost in iron stressed cells, since the phycocyanin peak at 625 nm is still present.

Room temperature and 77K fluorescence emission spectra provide a convenient way of determining the extent of energy transfer by phycobilisomes to reaction centres, and of chlorophyll fluorescence. Figures 6.2 and 6.3 represent room and low temperature emission spectra respectively, which show that fluorescence quenching by stressed cells expressing IsiA is not inhibited by phosphate. Energy transfer from phycobilisomes to the reaction centres is notably decreased, in particular to PSII upon illumination (black traces) compared with when they were in the dark (grey traces). There also appears to be a marked reduction in

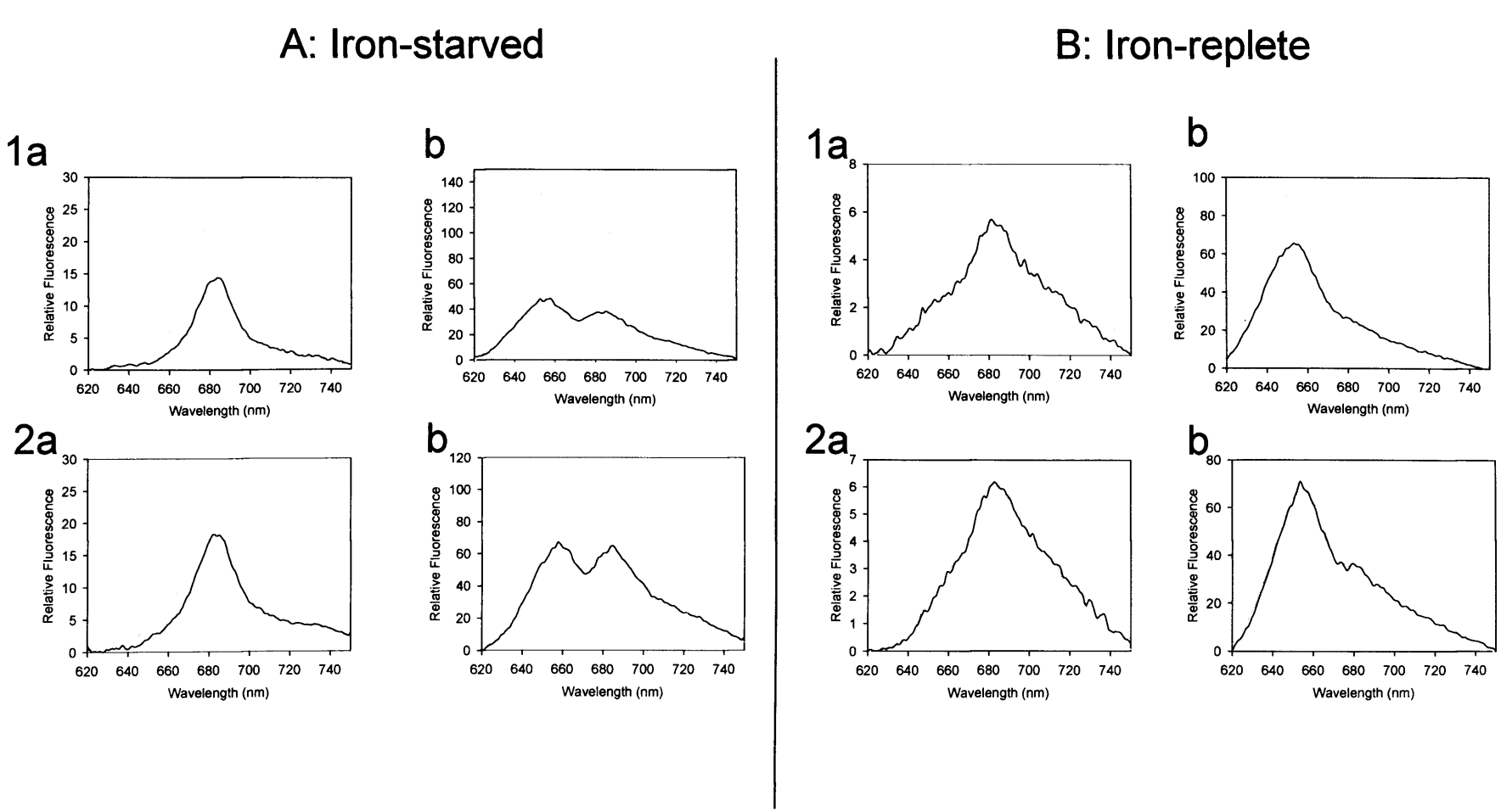


Figure 6.2. Room temperature whole cell fluorescence emission spectra for *S.* 6803 cells in the absence (1) or presence (2) of 1M phosphate buffer. Spectra depict excitation of chlorophyll at 435nm (a) or phycobilisomes at 600nm (b). Cells are dark incubated (grey) or quenching-induced by 5 minutes of white light incubation (black). Spectra were obtained using excitation and emission slit widths of 3nm and 5nm respectively.

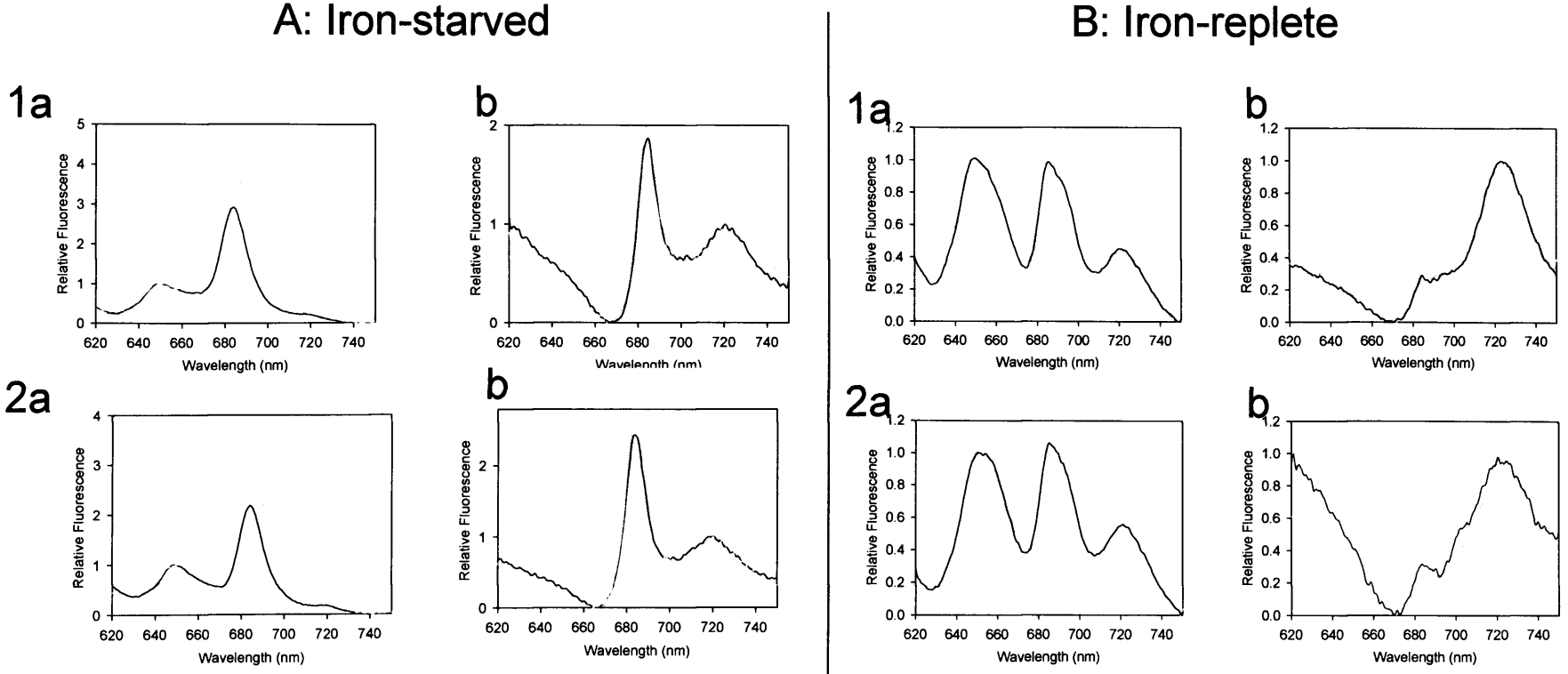


Figure 6.3. 77K whole cell fluorescence emission spectra for *S.* 6803 cells in the absence (1) or presence (2) of 1M phosphate buffer. Spectra depict excitation of phycobilisomes at 600nm (a) or chlorophyll at 435nm (b). Cells are dark incubated (grey) or quenching-induced by 5 minutes of white light incubation (black). Spectra were obtained using excitation and emission slit widths of 5nm.

fluorescence with chlorophyll excitation, especially evident from the room temperature traces. It is important to note that the phycobilisome quenching is approximately 63% compared with about 39% as observed with chlorophyll excitation. This suggests that phycobilisomes are, in part, responsible for NPQ of F_o.

The fluorescence decrease upon NPQ induction in the light and subsequent freezing gives final fluorescence still far higher than that observed in unstressed cells (figure 6.3B) which could either be attributed to IsiA or the terminal emitters from free phycobilisomes. Sadly, it is impossible to resolve which, as the fluorescence peak between 680 nm and 685 nm could be attributed to either one or both (Bibby *et al*, 2001; Glazer, 1984).

Figures 6.2B and 6.3B depict the corresponding room temperature and 77K emission spectra for non-stressed *Synechocystis* 6803 cells. A large amount of fluorescence quenching is predictably not observed in these cells. Phosphate treated and non-phosphate treated cells give similar spectra.

The fluorescence quenching by stressed cells may be owing to free phycobilisomes coupling to IsiA complexes in the thylakoid under saturating light conditions. Upon association with IsiA, the phosphate effect of intensifying the binding of the phycobilisomes prevents the recovery of fluorescence when the light source is removed. This is consistent with the fluorescence emission spectra in figure 6.3A.

6.4.3. Phycobilisome diffusion

Under normal physiological conditions, phycobilisomes are known to be highly mobile, travelling across the thylakoid membrane with diffusion coefficients of between 3 and 6 x 10^{-10} cm^2s^{-1} (Mullineaux *et al*, 1997; Sarcina *et al*, 2001; Aspinwall *et al*, 2004, for example), that in most circumstances PSII is immobile (Mullineaux *et al*, 1997; Sarcina *et al*, 2001) and that IsiA can diffuse (Sarcina and Mullineaux, 2004). These data were obtained using a technique known as Fluorescence Recovery After Photobleaching (FRAP), where fluorescent pigments are bleached out by a laser, and recovery over time being evidence for movement of those pigments within the cell. With elongated cells which have a regular arrangement of thylakoids, it is possible to quantify the rate of diffusion of

photosynthetic pigments like phycobilisomes and chlorophyll. This is not feasible in *Synechocystis* 6803, where spherical cells possess looping thylakoids (Nilsson *et al*, 1992). However, a qualitative determination of phycobilisome diffusion is possible. Following a bleaching treatment, the proportion of phycobilisomes able to fluoresce is markedly decreased, and a full recovery of fluorescence to the pre-bleached level is impossible (since the chromophore is irreversibly damaged), even if all the phycobilisomes are mobile. Typically, 30% of the total fluorescence was lost by conducting the bleach, so it was critical to allow for this in the measurement. Fluorescence levels were scaled to a theoretical level. This was effected in the following way: The total cell fluorescence was calculated before (pre) and after (time = 0s) the bleach from the fluorescence profiles in Sigmaplot 6.0. For each post-bleach profile, the fluorescence was scaled so that 100% recovery would signify that 100% of the remaining phycobilisome fluorescence was mobile. By analysing a series of images, fluorescence recovery can be determined as a proportion of the scaled total fluorescence in the bleached region before the bleach.

6.4.3.1. On BG11

Figures 6.4a and 6.4b clearly show that phycobilisomes of stressed and non-stressed cells on BG11 agar diffuse over a similar time-frame as that reported for in other cyanobacteria (Mullineaux *et al*, 1997; Sarcina *et al*, 2001; Aspinwall *et al*, 2004). In these figures, the bleached spot cannot be visualised as a line owing to both the very fast diffusion (faster than the time taken to record image 1) and the fact that the whole cell has been exposed to the high intensity laser. Imaging software can verify that in the centre of the bleach, the intensity is higher.

6.4.3.2. On phosphate

Light treatment of iron-stressed cells following a dark adaptation and phosphate treatment inhibits further mobility of phycobilisomes. This is shown in figure 6.5. Phycobilisomes of phosphate treated non-stressed cells do not move (data not shown).

By adsorbing phosphate-treated dark adapted stressed cells to agar and maintaining the dark conditions, it is possible to look at cells before and after the induction of quenching. Figure 6.6 demonstrates that dark adapted cells on phosphate have

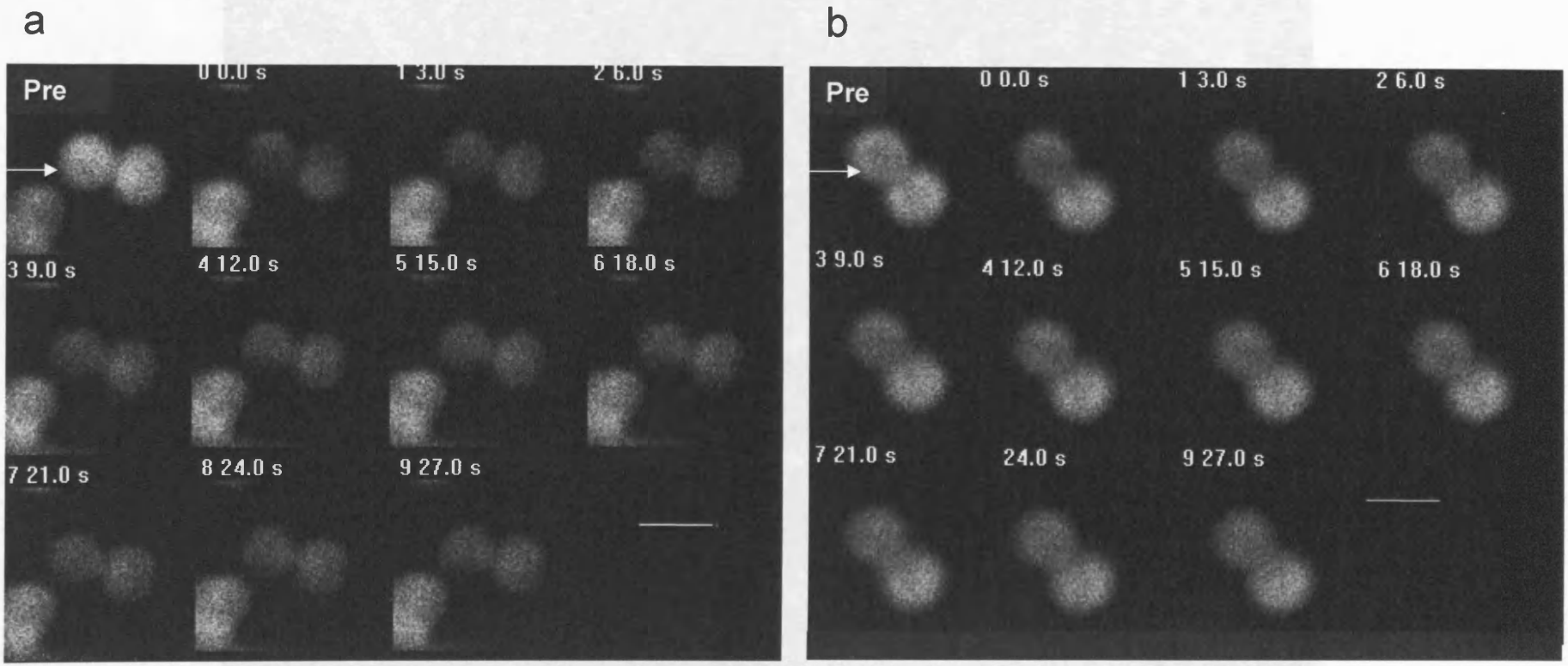


Figure 6.4. A series of images over 27s corresponding to a FRAP experiment of *S.* 6803 iron-starved cells (a) and iron replete cells (b) on BG11 agar. Fluorescence is from phycobilisomes. Scale bar = 5μm. Arrow denotes where the bleach will be most intense.

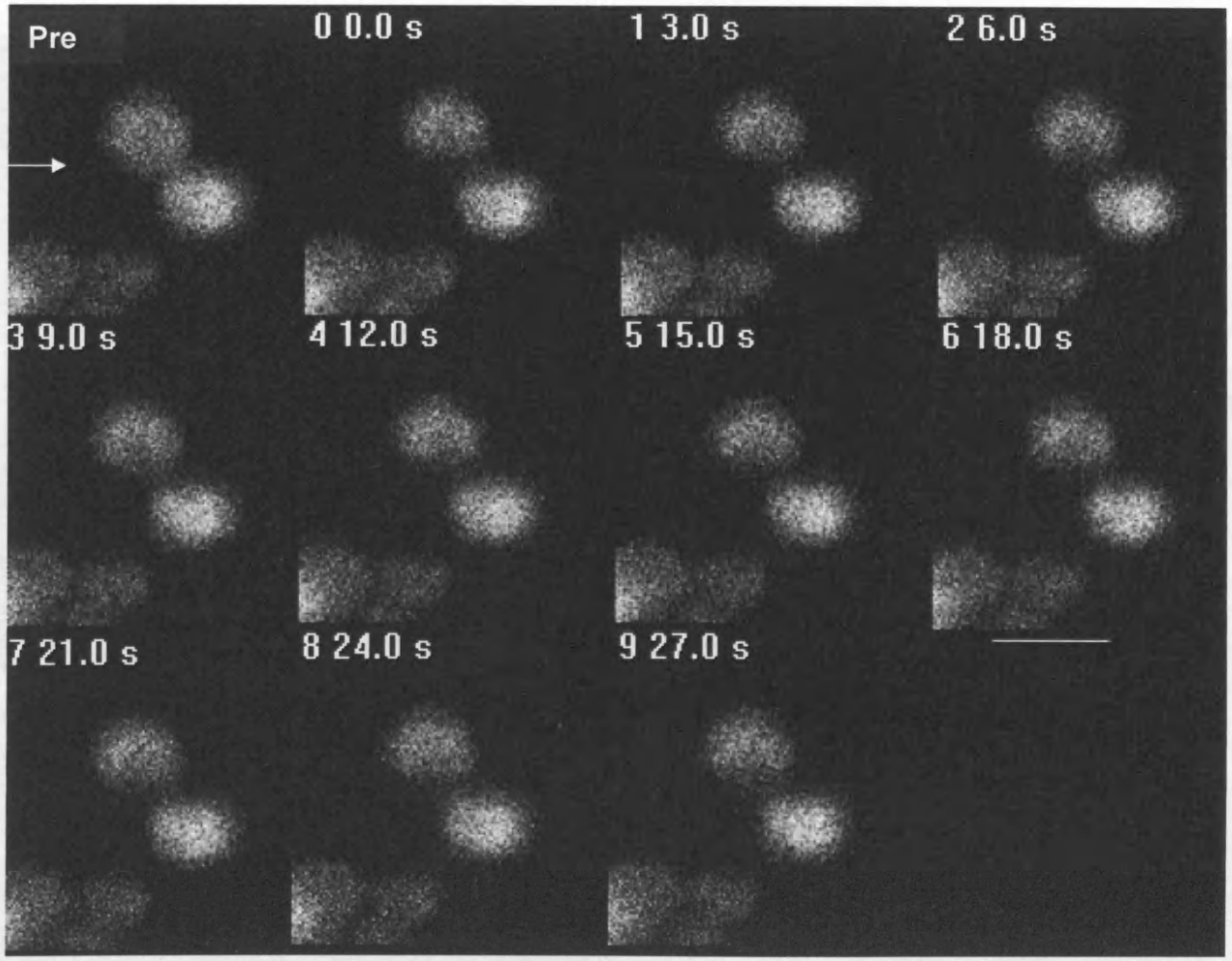


Figure 6.5. A series of images over 27s corresponding to a FRAP experiment of *S.* 6803 iron-starved cells on a 1M phosphate agar plate. Fluorescence is from phycobilisomes. Cells had been dark adapted in the presence of 1M phosphate buffer, light treated for 3 minutes and adsorbed to a 1M phosphate agar plate. Scale bar = 5μm. Arrow denotes where the bleach will be most intense.

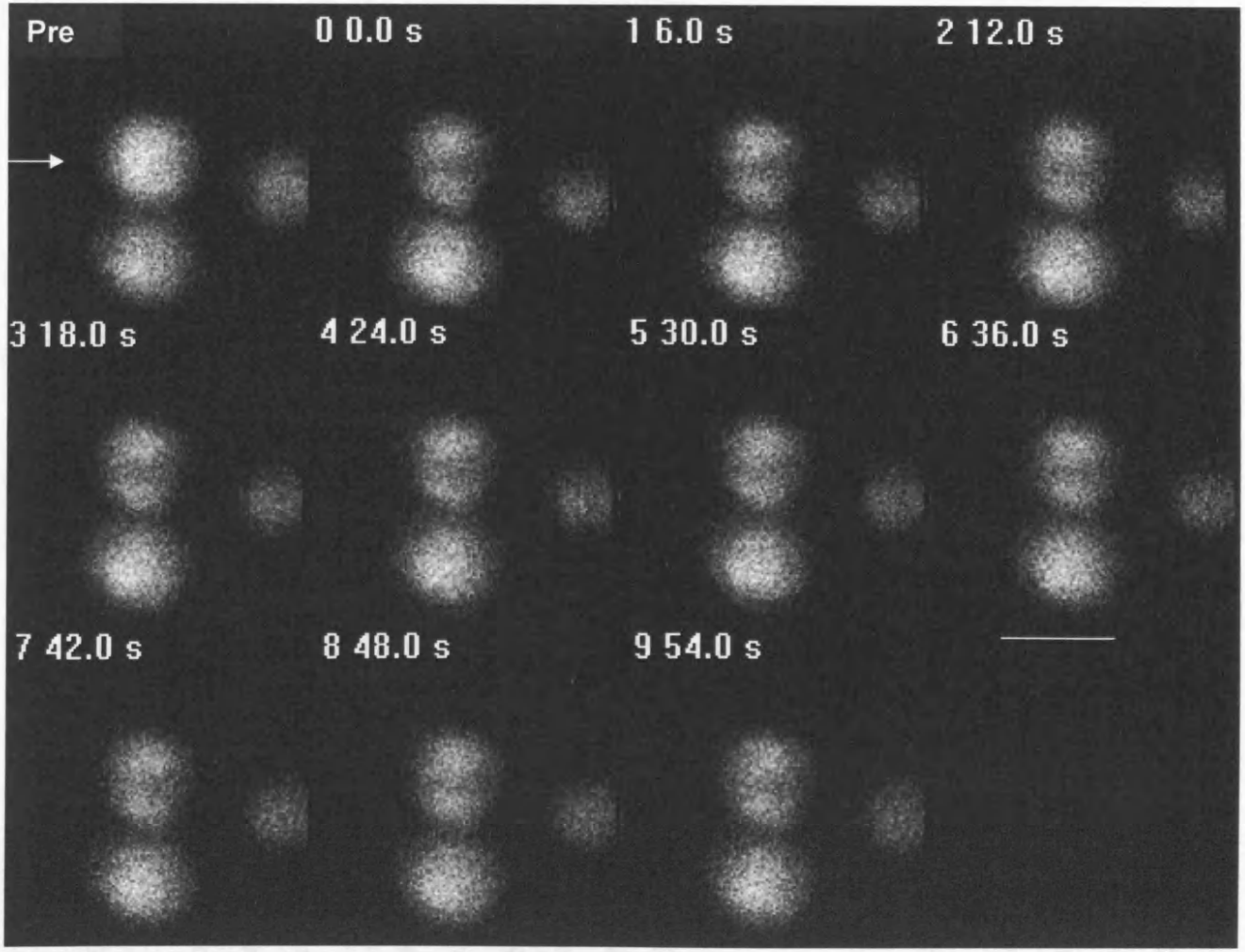


Figure 6.6. A series of images over 54s corresponding to a FRAP experiment of *S.* 6803 iron-starved cells on a 1M phosphate agar plate. Fluorescence is from phycobilisomes. Cells had been dark adapted in the presence of 1M phosphate buffer and adsorbed to a 1M phosphate agar plate. Scale bar = 5μm. Arrow denotes where the bleach will be most intense.

phycobilisomes still capable of diffusion. It has been shown that phycobilisomes of *Synechococcus* 7942 attached to reaction centres cannot diffuse when in phosphate concentrations above 0.3 M and that state transition fixation in *Synechocystis* 6803 correlated with the findings for *Synechococcus* 7942 (Joshua and Mullineaux, 2004). It may therefore be concluded that the movement observed here is attributable to free light harvesting complexes. Following a three minute induction of quenching of these cells under the microscope, phycobilisome diffusion cannot be detected in subsequent FRAP experiments (see figure 6.7). Non-stressed cells under the same conditions produce no detectable phycobilisome diffusion over the same time scales either before or after light treatment (data not shown).

Given that it is rather difficult to clearly see the partial recovery from the images, the recovery kinetics of a set of typical cells is shown in figure 6.8.

6.5. Discussion

The light harvesting complexes of cyanobacteria are capable of rapid diffusion on the thylakoid membrane (Mullineaux *et al*, 1997; Sarcina *et al*, 2001). This presumably enables them to efficiently distribute light energy to the photosynthetic reaction centres. While phycobilisomes are highly mobile, PSII is normally incapable of diffusion (Mullineaux *et al*, 1997; Sarcina and Mullineaux, 2004) and, where present, IsiA is only capable of very slow diffusion (Sarcina and Mullineaux, 2004). The FRAP technique has been employed in the case of both PSII and phycobilisomes to investigate the dynamics of fluorescent components within or on the surface of the membrane (Mullineaux *et al*, 1997; Sarcina *et al*, 2001, for example). Several studies have shown that phycobilisomes interact with PSI and PSII in a transient manner, allowing flexibility in light harvesting to the different reactions under differentially preferable illumination conditions (Rakhimberdieva *et al*, 2001; Mullineaux, 1994, for example).

The transient interactions of the phycobilisomes with the reaction centres are a prerequisite for the observation of state transitions in cyanobacteria. We have previously shown that this rapid term adaptation process involving changes the distribution of phycobilisomes to the two reaction centres (van Thor *et al*, 1998) is

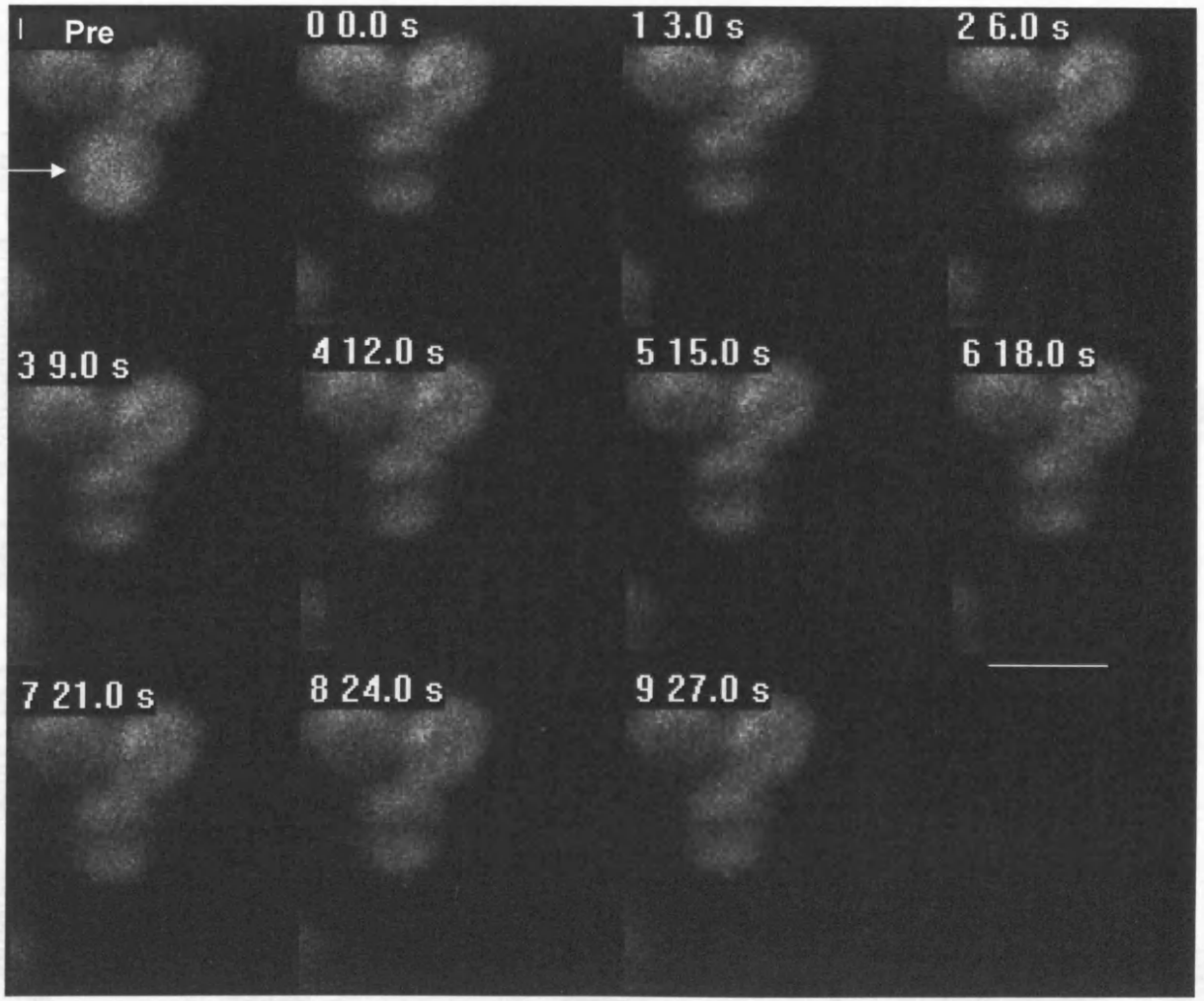


Figure 6.7. A series of images over 27s corresponding to a FRAP experiment of *S.* 6803 iron-starved cells on a 1M phosphate agar plate. Fluorescence is from phycobilisomes. Cells had been dark adapted in the presence of 1M phosphate buffer and adsorbed to a 1M phosphate agar plate. Following incubation for 3 minutes in the light, the experiment was undertaken. Scale bar = 5μm. Arrow denotes where the bleach will be most intense.

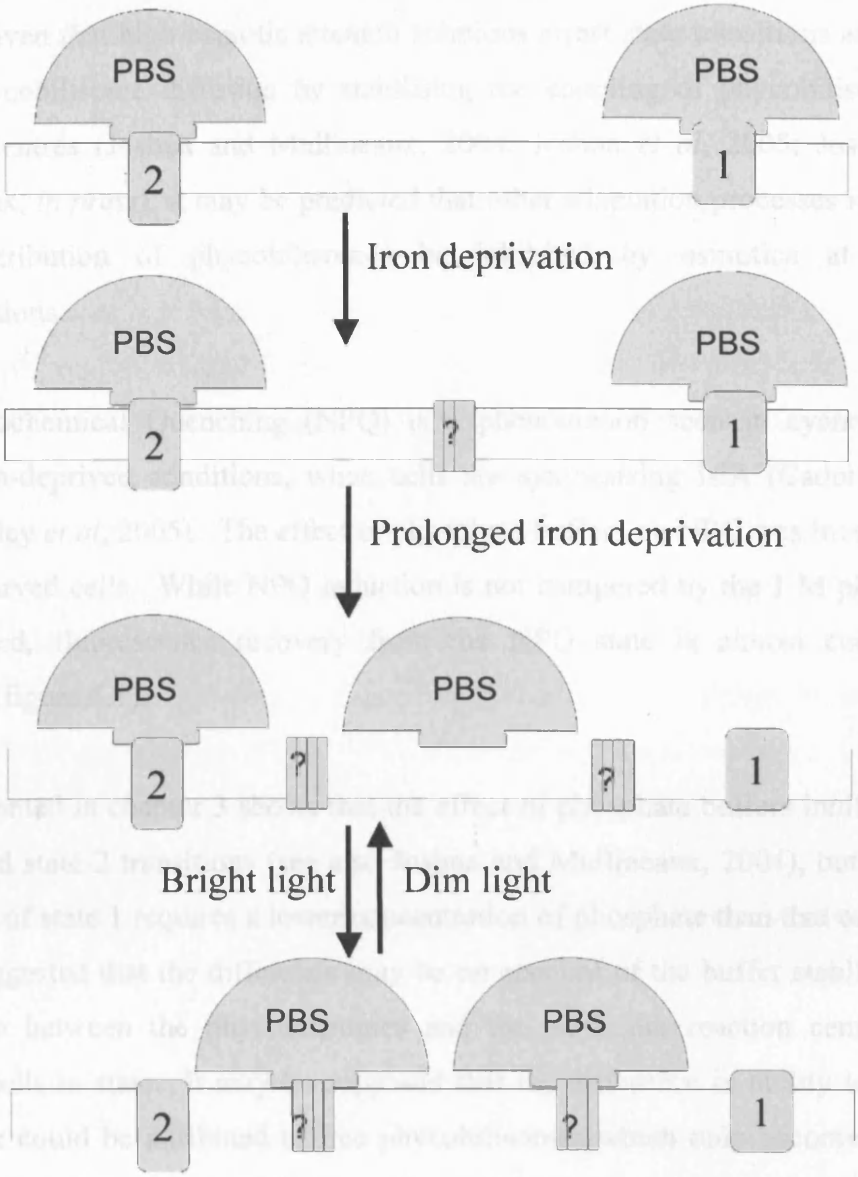


Figure 6.9. Cartoon of proposed mechanism for cyanobacterial NPQ
PBS = phycobilisome, 2 = PSII, 1 = PSI (trimer, monomer, or IsiA-containing supercomplex, ? = IsiA or other protein found in iron stressed cyanobacteria.

inhibited by immersing cells in high strength osmotica. The fixation of cells in state correlates with increased stability of phycobilisome-reaction centre interactions, determined by markedly decreased diffusion coefficients (Joshua and Mullineaux, 2004). Given that high-osmotic strength solutions arrest state transitions and long-range phycobilisome diffusion by stabilising the coupling of phycobilisomes to reaction centres (Joshua and Mullineaux, 2004; Joshua *et al*, 2005; Joshua and Mullineaux, *in press*), it may be predicted that other adaptation processes requiring the redistribution of phycobilisomes be inhibited by osmotica at similar concentrations.

Non-Photochemical Quenching (NPQ) is a phenomenon seen in cyanobacteria under iron-deprived conditions, when cells are synthesising IsiA (Cadoret *et al*, 2004; Bailey *et al*, 2005). The effect of phosphate buffers on NPQ was investigated in iron starved cells. While NPQ induction is not hampered by the 1 M phosphate buffer used, fluorescence recovery from the NPQ state is almost completely inhibited (figure 6.1).

Data presented in chapter 3 shows that the effect of phosphate buffers inhibits both state 1 and state 2 transitions (see also Joshua and Mullineaux, 2004), but that the inhibition of state 1 requires a lower concentration of phosphate than that of state 2. It was suggested that the difference may be on account of the buffer stabilising an interaction between the phycobilisomes and the particular reaction centre, thus "fixing" cells in state. It may be supposed that the difference in ability to fix the NPQ state could be attributed to free phycobilisomes which only become "fixed" upon coupling to complexes in the membrane following induction in the light, their interactions being stabilised by the buffer.

The fluorescence spectra depicted in figure 6.2 and 6.3 for phycocyanin excitation obtained at room temperature and 77K respectively, show very high fluorescence at 680-685nm in starved cells prior to the induction of NPQ. Upon illumination, and thus prompting quenching, the fluorescence decreases significantly. However, in comparison with non-stressed cyanobacteria, the fluorescence is still quite high. It may be supposed that this is on account of some decoupled phycobilisomes or alternatively phycobilisomes which are coupled and transferring energy directly to

IsiA complexes. Either way, it would be expected that there would be a corresponding fluorescence between 680 nm and 685 nm, which would correspond to the phycobilisome terminal emitters (Glazer, 1984) or the chlorophyll *a* component of IsiA (Bibby *et al*, 2001). Therefore neither the room temperature nor the low temperature spectra can be informative regarding which is causing the fluorescence.

FRAP measurements show that iron-starved cells possess some un-coupled phycobilisomes, as in 1 M phosphate, while phycobilisome diffusion is completely inhibited in iron-replete cells, a proportion of the phycobilisomes in iron-starved cells remain mobile. This suggests that they are not coupled to any membrane-integral protein complexes. (For cells in growth medium, the phycobilisomes diffuse rapidly both in iron-starved cells and in iron-replete cells (figure 6.4.) For comparison, the experiment was repeated following the induction of NPQ, and in this case, phycobilisome diffusion appears to be completely inhibited by 1 M phosphate (figure 6.5.). This correlates with the findings shown by the PAM fluorescence trace (figure 6.1.). This strongly suggests that there are some decoupled phycobilisomes in the iron-starved cell culture but neither in cultures grown under iron replete conditions nor in starved cells after NPQ induction. It may therefore be supposed that upon NPQ induction, the phycobilisomes interact directly with IsiA complexes in the thylakoid membrane.

IsiA mobility, while detectable, is much slower than that which is usually recorded for phycobilisome diffusion coefficients (Sarcina and Mullineaux, 2004), so it is likely that a phycobilisome-IsiA complex would be effectively immobile in the membrane, and therefore does not complicate the ability to conclude that:

a) prior to NPQ induction, phycobilisomes remain mobile despite the presence of phosphate buffer and then couple to IsiA, and

b) during the recovery period in the dark following induction of the quenched state, they remain immobile on account of their interaction with the chlorophyll complex being stabilised by the buffer.

Chlorophyll *a* fluoresces less than phycobilins owing to fluorescence being less efficient by the former. Further, competing processes remove more of the energy

absorbed by chlorophyll (or dissipate it as heat) more than for phycobilins. The radiative lifetime for chlorophyll a is roughly 13 ns (Borisov and Il'ina, 1971), but only 4 ns for phycobilins (Grabowski and Gantt, 1978). So, it would be expected that an uncoupled phycobilisome would have a far greater fluorescence yield than one that was attached to a chlorophyll *a*-containing complex like IsiA. Given this, it is possible that simply taking a proportion of free phycobilisomes and binding them to IsiA would prompt the observed fluorescence quenching. It cannot be discounted, however, that IsiA forms a specifically quenching state under these conditions (Cadoret *et al*, 2004), though the basis for this is undetermined. Fluorescence spectra at room temperature and 77K (figure 6.2 and 6.3 respectively) show that there is a chlorophyll effect as well as an effect on the phycobilins.

NPQ is rarely observed in cyanobacteria, except under conditions of severe oxidative stress caused by iron deprivation. Plants exhibit the capability for conducting NPQ under more diverse environmental conditions. This suggests that the underlying mechanisms for prokaryotic and eukaryotic NPQ are different. PSII is shielded from excess excitation in plants via quenching centres within their antenna complexes (Horton *et al*, 1996; Holt *et al*, 2004). Whilst this is the norm in eukaryotes, the prokaryotic process may not require the PSII antenna (except when IsiA is expressed under iron starvation), given that the quenching of F_0 involves free, uncoupled phycobilisomes, not those interacting with reaction centres.

Upon extreme iron starvation, cyanobacterial NPQ applies to both F_v and F_0 (Cadoret *et al*, 2004; Bailey *et al*, 2005). However, F_v quenching requires less extreme deprivation (Bailey *et al*, 2005). Under the conditions where F_0 occurs, it can be proposed that PSII antenna size is decreased on account of the light harvesting complexes interacting with IsiA in the membrane rather than the reaction centres resulting in quenching of F_v. As the deprivation is intensified, the proportion of free phycobilisomes is increased, and the proposed interaction of these with IsiA results in F_0 quenching (figure 6.9). The reason for why phycobilisomes have an increased affinity for IsiA and a corresponding decreased affinity for the reaction centre has yet to be elucidated, but will most certainly involve a signalling process, possibly triggered by blue light (Cadoret *et al*, 2004).

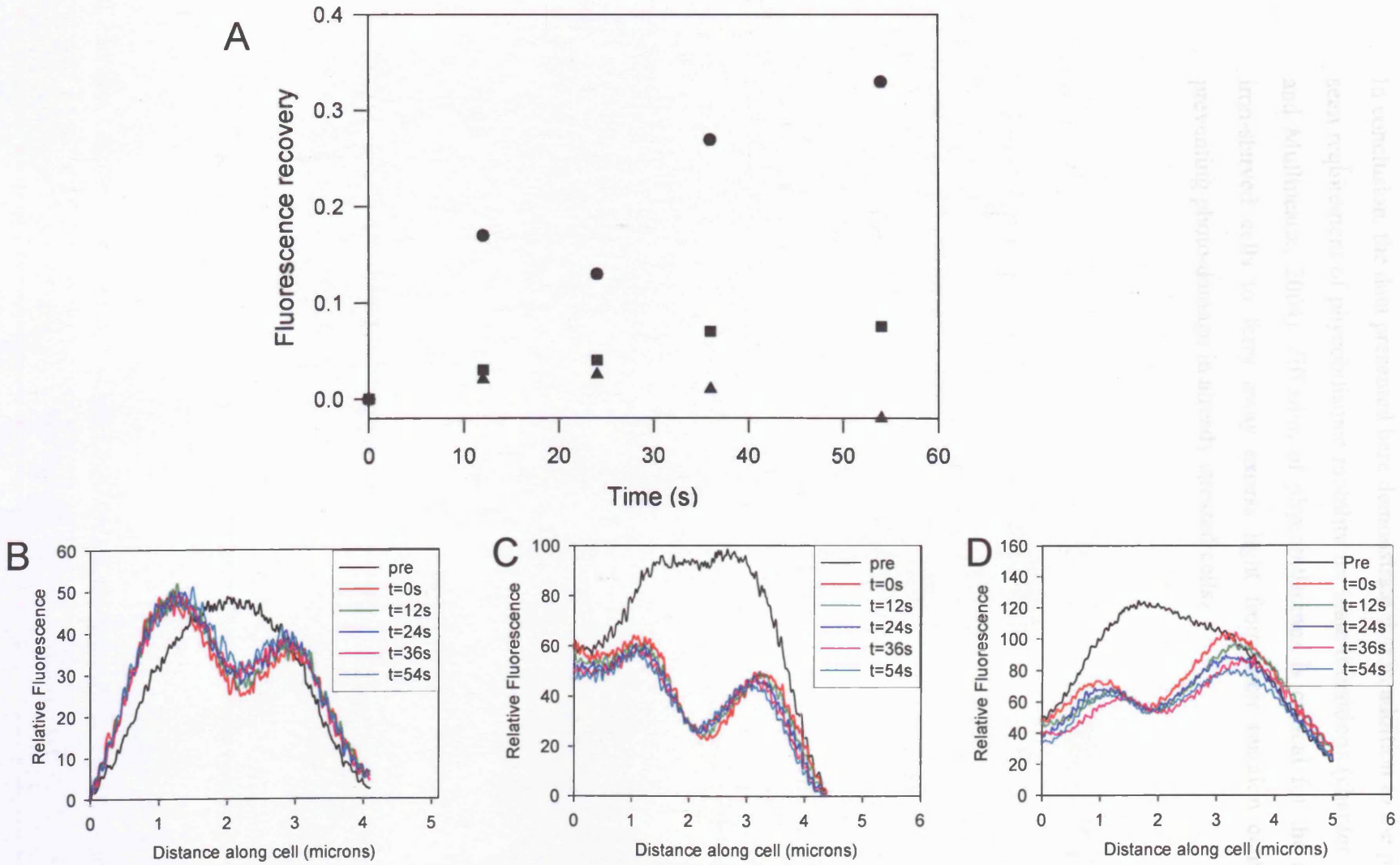


Figure 6.8. Recovery kinetics for typical cells (A). Circles correspond to FRAP of an iron starved cell pre-illumination (before induction of NPQ), squares depict a cell post-NPQ. Triangles represent recovery kinetics of a non-stressed cell in 1M phosphate. The recovery was calculated from fluorescence profiles given by B, C and D respectively.

In conclusion, the data presented here demonstrate that in addition to the previously seen requirement of phycobilisome mobility for state transitions (chapter 3; Joshua and Mullineaux, 2004), diffusion of phycobilisomes is critical for the ability of iron-starved cells to ferry away excess light from their reaction centres, thus preventing photo-damage in already stressed cells.

Chapter 7: Psb28 - A protein involved in maintaining PSII organisation in the thylakoid membrane?

Chapter 7: Psb28 - A protein involved in maintaining PSII organisation in the thylakoid membrane?

7.1. Objective

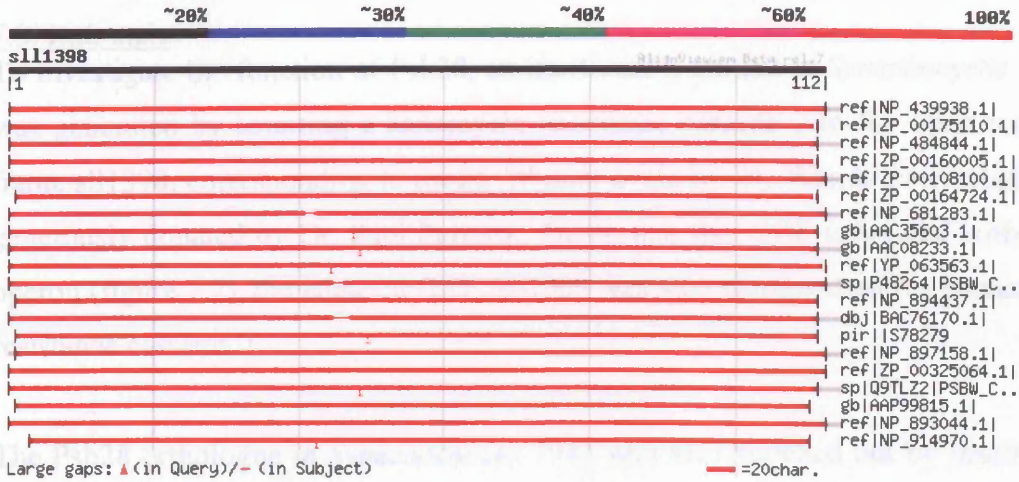
- To determine the function of *psb*28, a gene conserved within phototrophic organisms

7.2. Introduction

Psb28, sometimes referred to as PsbW and Ycf79, is a 12kDa protein conserved across photosynthetic organisms (Reardon and Price, 1995) (figure 7.1.). The protein is distinct from the PSII subunit also called PsbW. The *psb*28 gene was first identified in the chloroplast genome of the red alga *Porphyrium cruentum* and an orthologue in *Synechocystis* 6803 was identified from preparations of PSII. In photosynthetic eukaryotes, *Chlamydomonas reinhardtii* and *Arabidopsis thaliana* included, the gene is present in the nuclear genome. The eukaryote homologues encode proteins with an N-terminal extension sequence corresponding to a chloroplast targeting sequence.

The protein contains no membrane-spanning domains, and evidence that the 12kDa phosphoprotein co-purifies with PSII spinach preparations (Lindahl *et al*, 1995) suggests it is located stromally in chloroplast thylakoids. A protein of similar size and properties was extracted in *Synechocystis* 6803 (Kashino *et al*, 2002). In PSII extractions from other organisms, *Thermosynechococcus elongatus* included, the protein does not always co-purify (Ferreira *et al*, 2004). This may be as a result of the protein being extrinsic and thus lost during the purification. BLAST searching of Cyanobase (http://www.kazusa.or.jp/cyano) clearly demonstrates that the gene (ORF tlr0493) is present in this organism (figure 7.1.).

Some cyanobacteria, for example the relatively closely related species *Synechocystis* 6803 and *Anabaena* 7120, contain a second copy of the gene, less similar to the well conserved first copy. The photosynthetic role of the Psb28 has not been established as yet, but its conservation across a diverse array of species suggests it plays an important role. The second copy is redox responsive, as determined using microarray analysis using DCMU or DBMIB (Hihara *et al*, 2003).



Sequences producing significant alignments:	(bits)	Value
ref|NP_439938.1| photosystem II 13 kD protein [Synechocystis sp....	224	4e-58
ref|ZP_00175110.1| hypothetical protein Cwat026023 [Crocosphaera...	164	5e-40
ref|NP_484844.1| photosystem II protein W [Nostoc sp. PCC 7120] ...	152	1e-36
ref|ZP_00160005.1| hypothetical protein Avar330001 [Anabaena var...	152	1e-36
ref|ZP_00108100.1| hypothetical protein [Nostoc punctiforme]	151	3e-36
ref|ZP_00164724.1| COG1187: 16S rRNA uridine-516 pseudouridylate...	143	7e-34
ref|NP_681283.1| photosystem II reaction center W protein [Therm...	141	3e-33
gb|AAC35603.1| PSII protein W [Guillardia theta] gi|11467617|ref...	131	4e-30
gb|AAC08233.1| Photosystem II protein W [Porphyra purpurea] gi|2...	130	5e-30
ref|YP_063563.1| conserved hypothetical plastid protein [Gracila...	129	2e-29
sp|P48264|PSBW_CYAPA Photosystem II reaction center W protein gi...	128	2e-29
ref|NP_894437.1| possible Photosystem II reaction center Psb28 p...	125	2e-28
dbj|BAC76170.1| photosystem II protein W [Cyanidioschyzon merola...	124	3e-28
pir||S78279 photosystem II protein W, 13K - Odontella sinensis c...	124	3e-28
ref|NP_897158.1| putative photosystem II reaction center Psb28 p...	122	2e-27
ref|ZP_00325064.1| hypothetical protein Tery02005150 [Trichodesm...	120	6e-27
sp|Q9TLZ2|PSBW_CYACA Photosystem II reaction center W protein gi...	114	3e-25
gb|AAP99815.1| Photosystem II reaction centre W protein [Prochlo...	114	6e-25
ref|NP_893044.1| possible Photosystem II reaction center Psb28 p...	111	4e-24
ref|NP_914970.1| photosystem II protein W-like protein [Oryza sa...	111	4e-24

Figure 7.1. Conservation of Psb28 in phototrophs
(http://www.kazusa.or.jp/cyano)

Several sequences show homology to the Psb28 translated sequence. This is a tblastn search of the amino acid sequence of Psb28. High "bit" scores and low "expected" values correspond to a low probability that such conservation between the search sequence and the BLAST-retrieved sequence occurs by chance. The sequences highlighted in red correspond to the *Synechocystis* 6803 and *Thermosynechococcus elongatus* (close relative of *Synechococcus* 7942) sequences. Probing the JGI website (http://genome.jgi-psf.org/draft_microbes/synel.home.html) using the latter sequence derived from here extracted the *psb*28 gene insertionally inactivated in this chapter.

7.3. Rationale

To investigate the function of Psb28, an inactivation mutant in *Synechocystis* 6803 was generated by inserting a kanamycin resistance cassette into the open reading frame sll1398, corresponding to psb28 (Mutant made by Dr. Wendy Fairclough and generously donated by Dr. Saul Purton). Given that this ORF is located within an operon (figure 7.2), the adjacent ORF, sll1399 was also disrupted with a kanamycin resistance cassette.

The Psb28 orthologue in *Synechococcus* 7942 was also knocked out by insertional inactivation. The two species of cyanobacterium used for this study have different advantages. *Synechocystis* 6803 is glucose tolerant, and so, should Psb28 be critical to photosynthetic function, an otherwise lethal mutant might be rescued. *Synechococcus* 7942 permits the determination of diffusion coefficients of integral fluorescent components of the photosynthetic apparatus using the FRAP technique, whilst *Synechocystis* 6803 permits only a qualitative estimation of the dynamics of such components. Future work may focus on determining the dynamics of Psb28 itself, so GFP fusions would be desirable in both species. Given that no GFP fusions have successfully been generated in *Synechococcus* 7942, work in *Synechocystis* 6803 inactivation mutants is critical for control purposes.

7.4. Results

7.4.1. Genotypic Characterisation of the *Synechocystis* 6803 *psb28*$^-$

The *psb28* gene of *Synechocystis* 6803 is located within an operon. The gene was cloned into pBluescript, and the 1.4kb kanamycin resistance cassette from the HincII-digested pUC4K plasmid inserted into an internal *Afe*I site (Wendy Fairclough). To confirm that the phenotype of the mutant generated by this construct was specific to this gene being inactivated, the adjacent open reading frame, sll1399 was cloned and interrupted with a kan^R cassette with *Eco*R1 ends at an internal *Mfe*I site (Saul Purton). The constructs are shown in figure 7.3.

The *psb*28 gene in *Synechococcus* 7942 was located via BLAST searching of the JGI website. The 0.9kb PCR product was cloned into pBluescript. It was digested with *Msc*I and *Afe*I, deleting ~200bp, and the *Hinc*II-kanR cassette was inserted

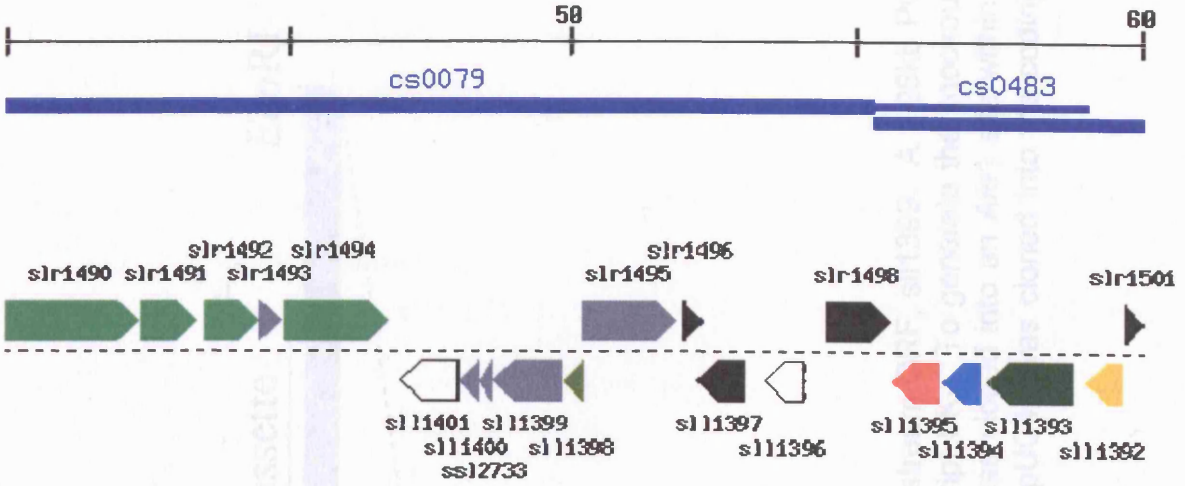


Figure 7.2. The *psb*28 gene (sll1398) is located at the head of a cluster of five open reading frames. This map was obtained from the Cyanobase website (http://www.kazusa.or.jp/cyanobase).

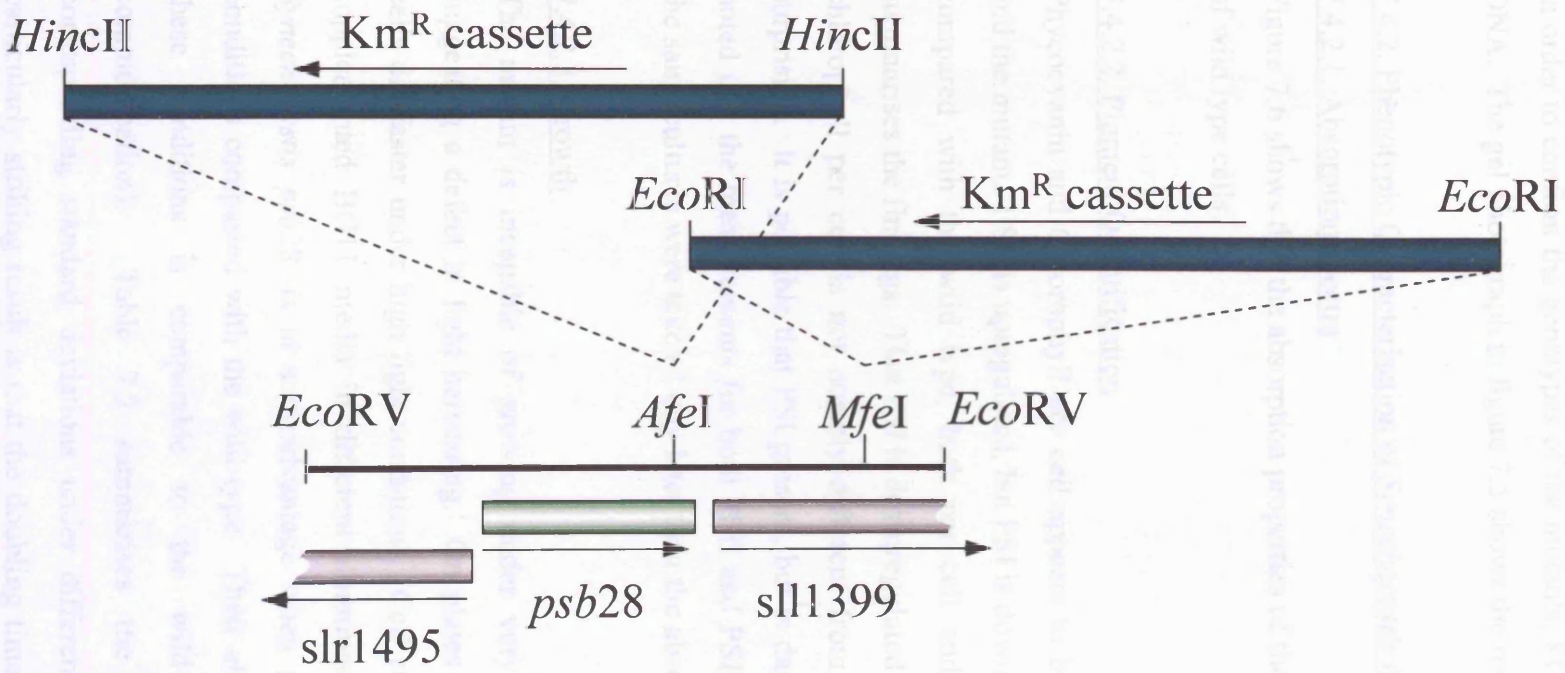


Figure 7.3. Knockout constructs for *Synechocystis* 6803 *psb*28 (*ycf*79) and downstream ORF, sll1399. A 1.06kb PCR product containing slr1495, psb28 and sll1399 was cloned into the EcoRV site of pBluescript SK-. To generate the knockout of *psb*28, the *Hinc*II fragment of the kanamycin resistance cassette excised from pUC4K was cloned into an *Afe*1 site within the *psb*28 coding region. The *Eco*R1 flanked kanamycin resistance cassette excised from pUC4K was cloned into the coding region of sll1399 to generate the knockout of a gene downstream of *psb*28.

(Saul Purton). Transformants of the cyanobacterium were selected on BG11 supplemented with kanamycin, and repeatedly re-streaked to encourage segregation. The construct is depicted in figure 7.4.

In order to confirm the genotypes of the mutants, PCR was undertaken on genomic DNA. The gel photograph in figure 7.5 shows the results.

7.4.2. Phenotypic Characterisation of *Synechocystis* 6803 *psb*28$^-$

7.4.2.1. Absorption spectra

Figure 7.6 shows that the absorption properties of the mutant are comparable to that of wild type cells.

7.4.2.2. Pigment Quantification

Phycocyanin and Chlorophyll per cell appears to be comparable in the wild type and the mutant. PSII is upregulated, but PSI is down by a factor of 10 in the mutant compared with the wild type, both per cell and per chlorophyll. Table 7.1 summarises the findings. That PSI is downregulated by so much but that the overall chlorophyll per cell is not notably different from the wild type (figure 7.6) is surprising. It is possible that PSI present, but is damaged. However, it should be noted that the measurements for both PSII and PSI quantification, while taken on the same cultures were taken 1 day later than the absorption spectra.

7.4.2.3. Growth

The mutant is incapable of growing under very dim illumination conditions, suggesting a defect in light harvesting. On plates and in liquid medium, mutant cells die faster under high light conditions. Cell cultures spotted onto differently supplemented BG11 media in different illumination conditions suggested that *Synechocystis psb*28$^-$ is at a disadvantage when grown under low illumination conditions comparted with the wild type. Their ability to grow in glucose under these conditions is comparable to the wild type (S. Purton, personal communication). Table 7.2 summarises the mean doubling times with corresponding standard deviations under different illumination conditions. A particularly striking result is that the doubling time for growth under blue light is significantly different, with wild type cells doubling in 71.67±1.53 hours and the

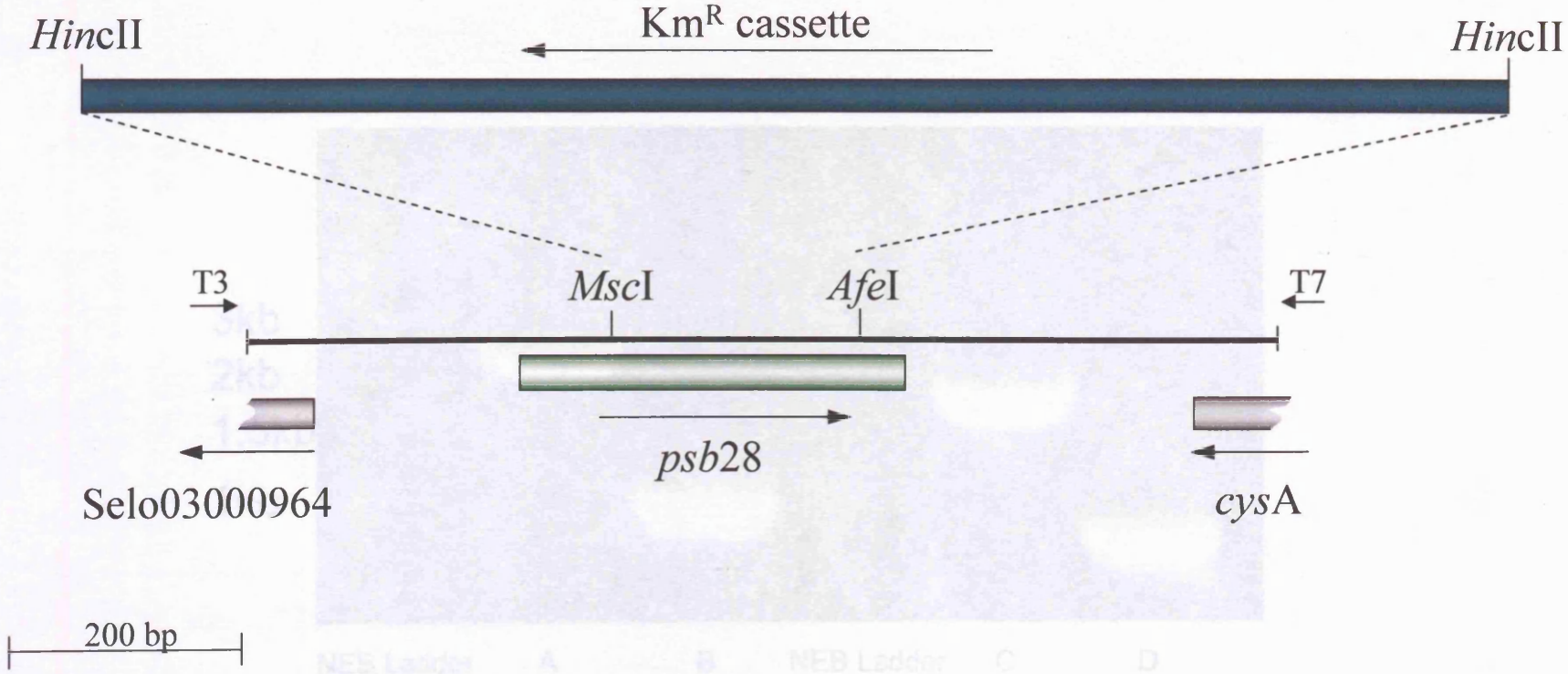


Figure 7.4. Knockout construct for *Synechococcus* 7942 *psb*28^{-} mutant. A 0.9kb PCR product containing *psb*28 coding region was cloned into an *Eco*RV/*Hinc*II double digested pBluescript sk-. The *Hinc*II flanked kanamycin resistance cassette was cloned into that *Msc*I/*Afe*I digested plasmid, interrupting the partially deleted coding region.

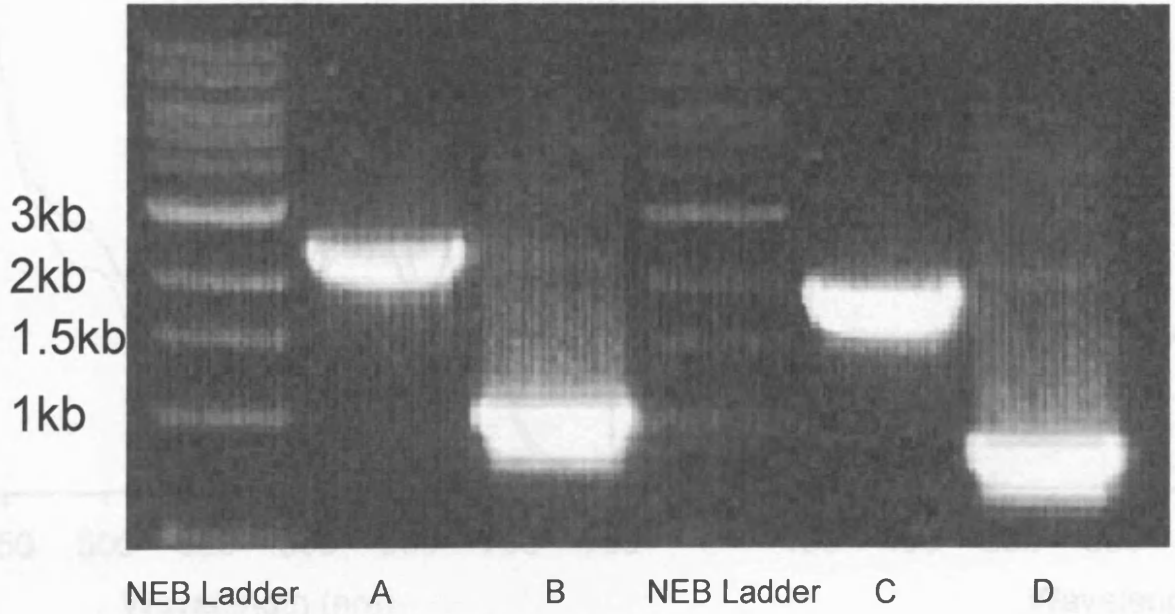


Figure 7.5. Gel photograph showing PCR from a) genomic DNA of the sll1399 mutant of *Synechocystis* 6803, b) the plasmid construct transformed into *Synechocystis* 6803 to obtain sll1399$^-$, c) genomic DNA of *Synechococcus* 7942 *psb*28$^-$ and d) the plasmid construct transformed into *Synechococcus* 7942 to obtain *psb*28$^-$. NEB 1kb Ladder is shown as a size marker.

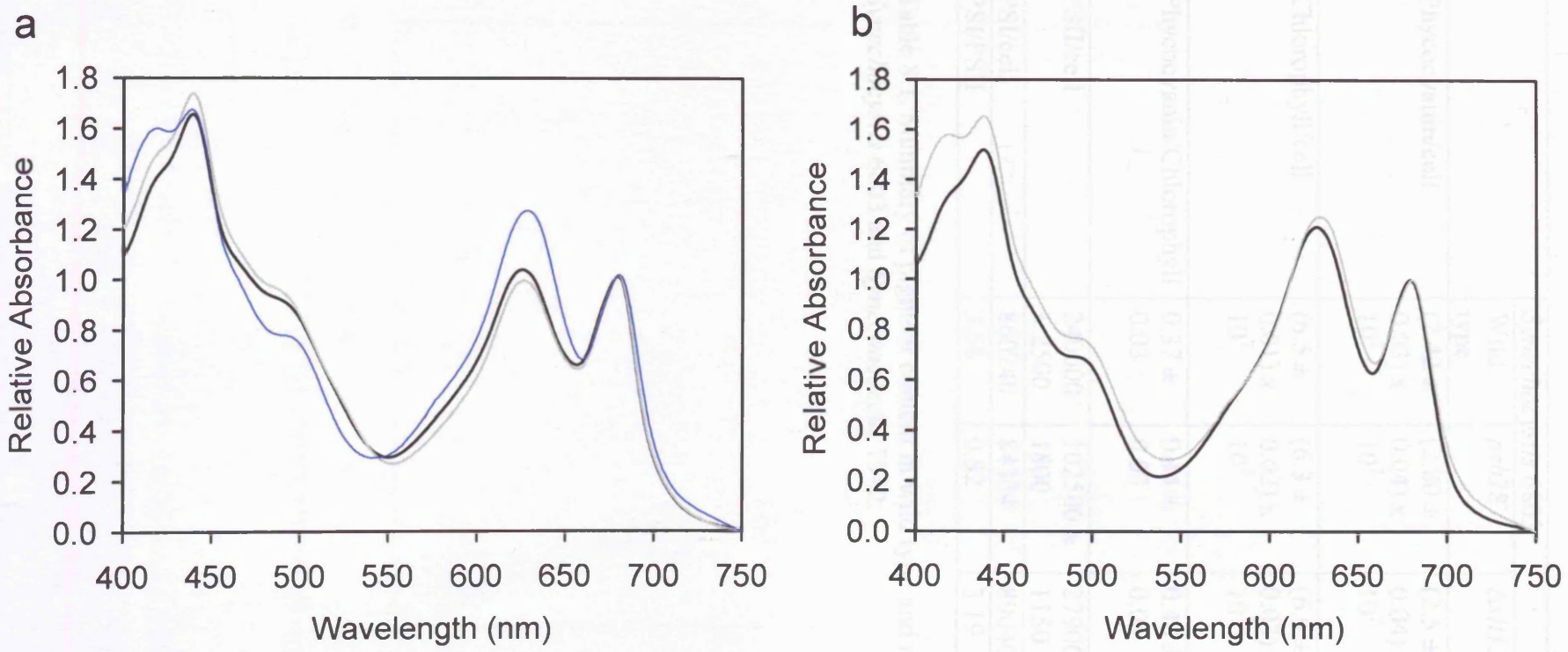


Figure 7.6. Absorption spectra for *Synechocystis* 6803 GT wild type (black), *psb*28^{-} (grey) and sll1399^{-} (blue). Cells were normalised to the peak corresponding to chlorophyll a at 678nm. 8.5b are the corresponding spectra for *Synechococcus* 7942 wild type (black) and *psb*28^{-} (grey).

	Synechocystis 6803			*Synechococcus* 7942	
	Wild type	*psb28*$^-$	Δ*sll1399*	Wild type	*psb28*$^-$
Phycocyanin/cell	(2.42 ± 0.03) x 10^7	(2.80 ± 0.04) x 10^7	(2.5 ± 0.04) x 10^7	(6.50 ± 0.04) x 10^7	(6.24 ± 0.05) x10^7
Chlorophyll/cell	(6.5 ± 0.01) x 10^7	(6.3 ± 0.02) x 10^7	(6.9 ± 0.03) x 10^7	(1.00 ± 0.02) x 10^8	(1.03 ± 0.06) x10^8
Phycocyanin/Chlorophyll	0.37 ± 0.03	0.44 ± 0.03	0.40 ± 0.04	0.65 ± 0.01	0.61 ± 0.04
PSII/cell	240000 ± 2500	102500 ± 1800	279000 ± 1150	160000 ± 2400	130000 ± 4500
PSI/cell	860240	84334	890400	488000	320000
PSI/PSII	3.58	0.82	3.19	3.05	2.46

Table 8.1. Summary of pigment content in wild type and mutant strains of *Synechocystis* 6803 and *Synechococcus* 7942.

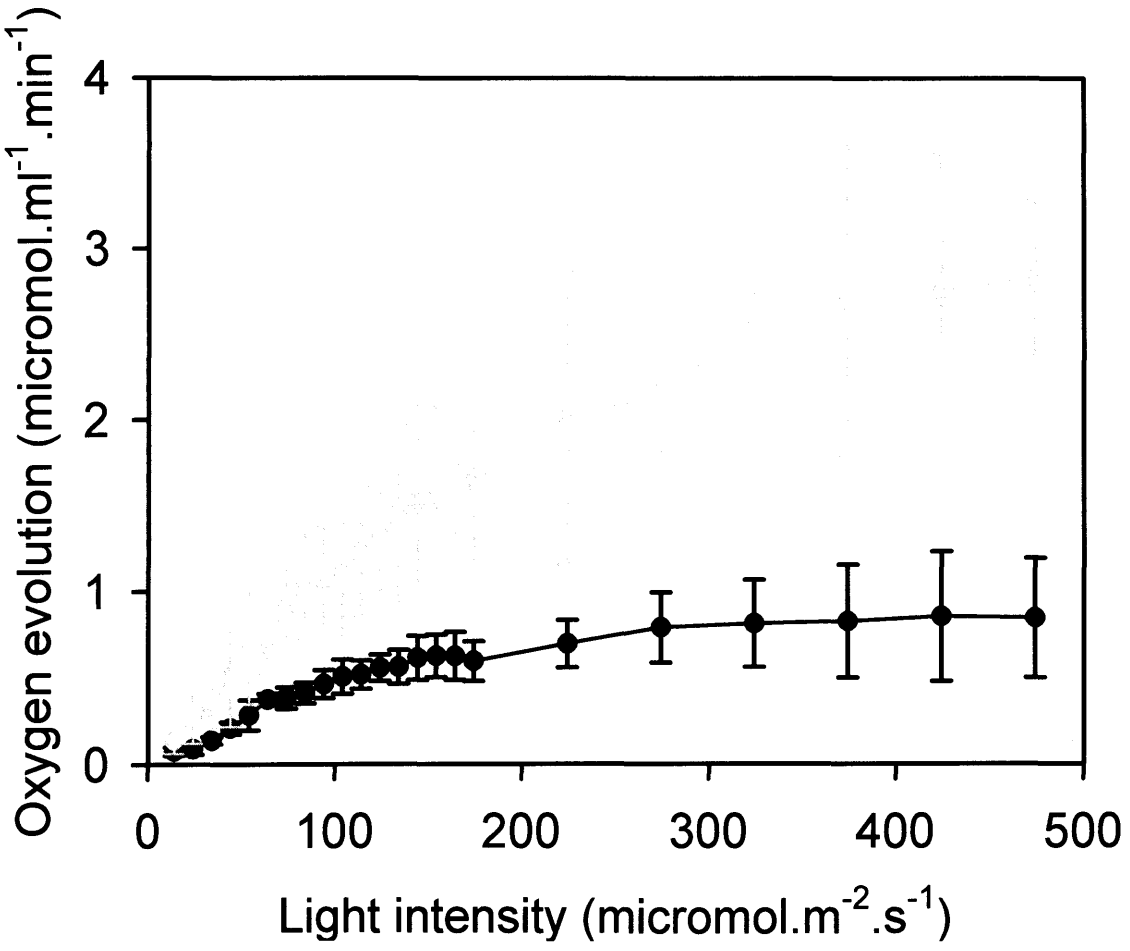


Figure 7.7 shows oxygen evolution per chlorophyll for wild type *S.* 6803 (black) and *S.* 6803 *psb*28^{-} (grey). Cells had been grown under normal illumination conditions.

Synechocystis 6803 *psb*28$^-$ mutant doing so in 114.33±3.06 ($t = 21.64$, $p<2.7 \times 10^{-5}$). As blue light is not phycobilin-absorbed, this result is suggestive of the photosystem chlorophylls in the mutant harvesting light sub-optimally.

	Strain				
Illumination conditions	*Synechocystis* 6803			*Synechococcus* 7942	
	Wild type	*psb*28$^-$	Δ*sll1399*	Wild type	*psb*28$^-$
High	12±2	15±1	13±1	12±4	13±3
Moderate	15±3	18±2	14±2	15±5	19±2
Low	30±2	50±5	29±6	45±2	72±5
Blue	71±3	114±2	81±6	75±3	101±3

Table 7.2. Summary of the doubling times (hours) for the wild type and mutant strains in *Synechocystis* 6803 and *Synechococcus* 7942 under different illumination conditions.

7.4.2.4. Oxygen Evolution

The mutant is evolving oxygen, confirming that PSII is functioning and that the electron transport is occurring. Oxygen evolution is significantly up-regulated in the mutant ($t = 5.25$, $p < 2.98 \times 10^{-6}$). This is consistent with the fact that PSII per cell is increased relative to the wild type. Figure 7.7 depicts oxygen evolution for 3 wild type cultures of *Synechocystis* 6803 and 3 *psb*28$^-$ mutant cell cultures grown under moderate light intensity. For the purposes of the measurement, cells were provided with increasing light intensities until saturation. These data show that not only is there increased oxygen evolution in the mutant, but that the mutant cells reach light saturation later than the wild type cells.

7.4.2.5. State Transitions

Mutant cells are capable of state transitions under moderate and high illumination conditions used, while wild type cells are classically capable of this adaptation particularly when grown in low intensity illumination, but also up to and including 70 $\mu molm^{-2}s^{-1}$ (high light). This deduction was made using room temperature fluorescence emission time-course measurements (figure 7.8).

However, the state transitions are faster in the mutant (figure 7.8c) compared with the wild type (figure 7.8a). In the mutant, the transition to state 1 typically took place in 1 minute, compared with a typical 5 minutes for the wild type. Additionally, and unlike in the wild type where 77K emission spectra show differences when exciting chlorophyll *a* as well as phycobilisomes (the latter shown in figure 7.9), the mutant cells lose the chlorophyll effect usually observed (figure 7.10). That is, there appears to be no increased fluorescence from PSII relative to PSI for cells frozen in state 1 compared with state 2 (figure 7.10).

Further, there appears to be a small amount of fluorescence quenching after the occurrence of the transition to state 1 (see figure 7.8c), possibly owing to a de-coupling of some phycobilisomes (see 77K emission spectra for frozen suspensions at 3 minutes post-provision of the red actinic light source). This observation provides a strong indication that Psb28 is required for efficient coupling (or de-coupling) of phycobilisomes to reaction centres.

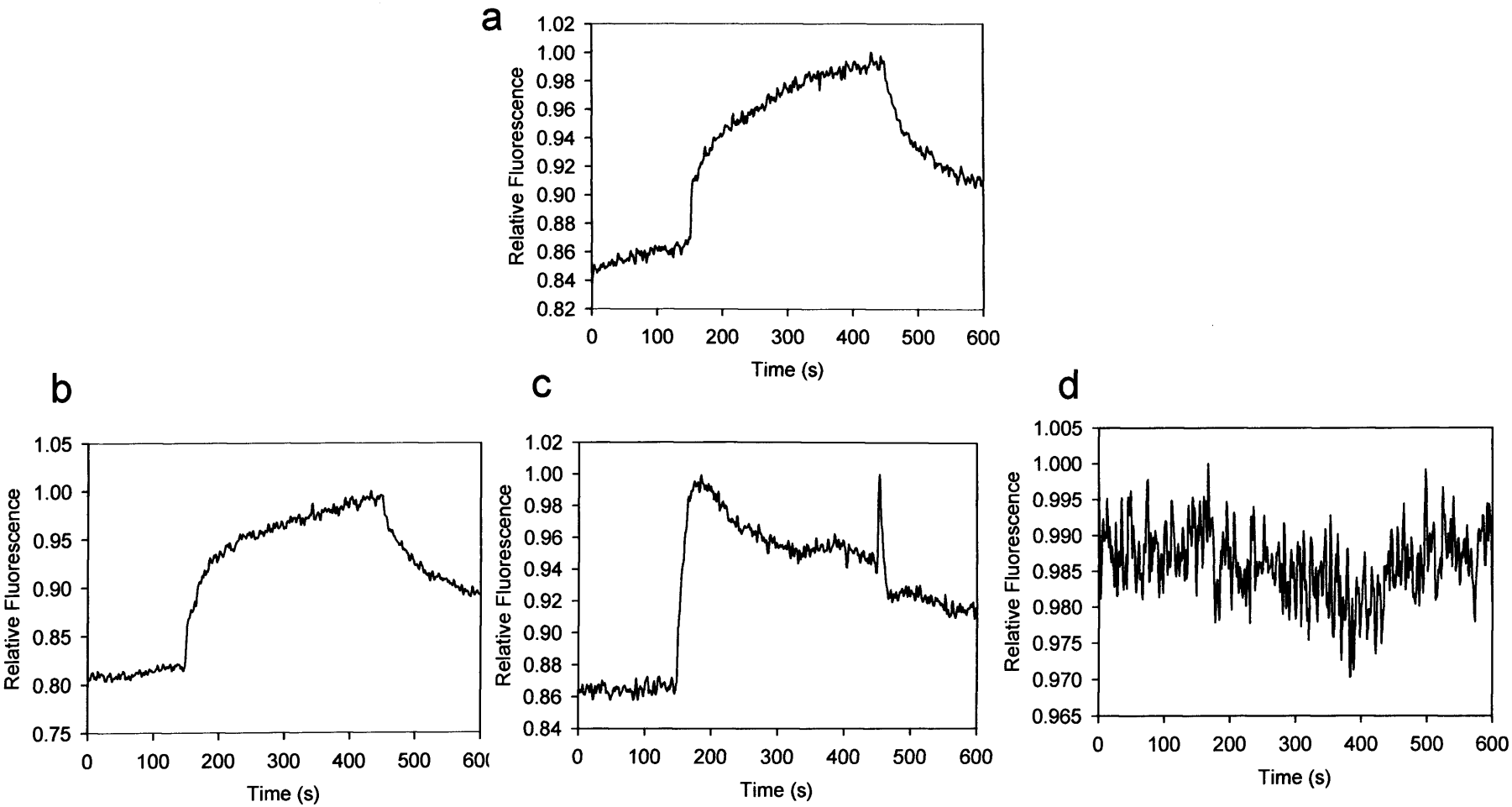


Figure 7.8 shows a room temperature time-course, for excitation of phycobilisomes at 600nm and emission at 680nm, corresponding to PSII. Traces presented are for moderately grown *Synechocystis* 6803 GT wild type (a) and, under high (b), moderate (c) and low (d) light, *psb*28^{-}. Cells were grown under high light conditions. Spectra were obtained using cells concentrated to 5μM chlorophyll, with excitation and emission slit widths at 10nm and 5nm respectively. Red illumination was provided between 150s and 450s, otherwise cells received dim yellow illumination.

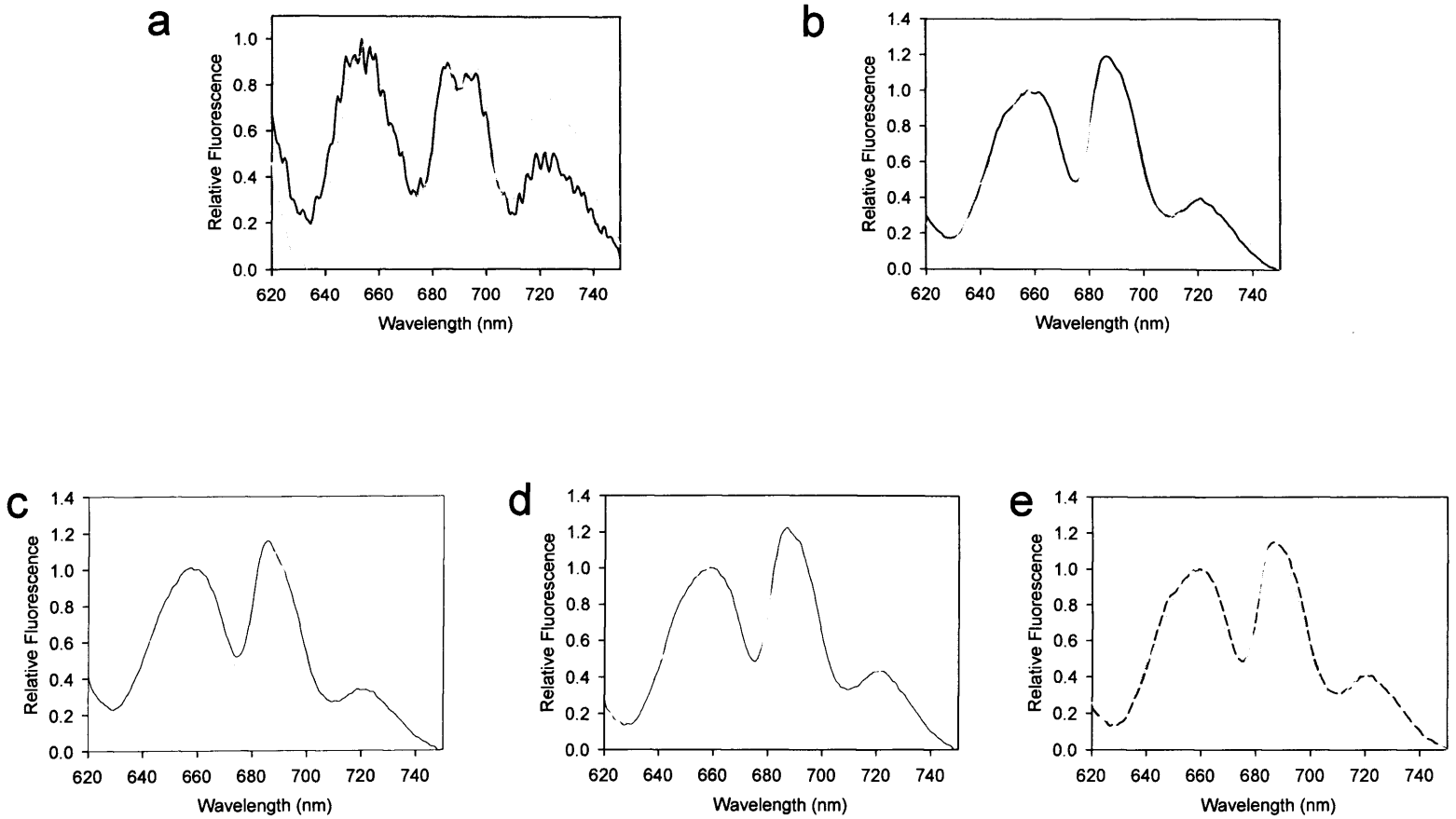


Figure 7.9: 77K whole cell fluorescence emission spectra for excitation of phycobilisomes at 600nm of light (black) or dark (grey) adapted *Synechocystis* 6803. a) wild type b) *psb*28^{-}. c) and d) are also *psb*28^{-}, but the light adaptation time is 1 minute and 3 minutes respectively. e) corresponds to mutant spectra where the black line represents cells adapted for 3 minutes in red light and then 1 minute in darkness. Spectra were obtained using excitation and emission slit widths of 5nm, and were normalised to phycocyanin fluorescence at 650nm.

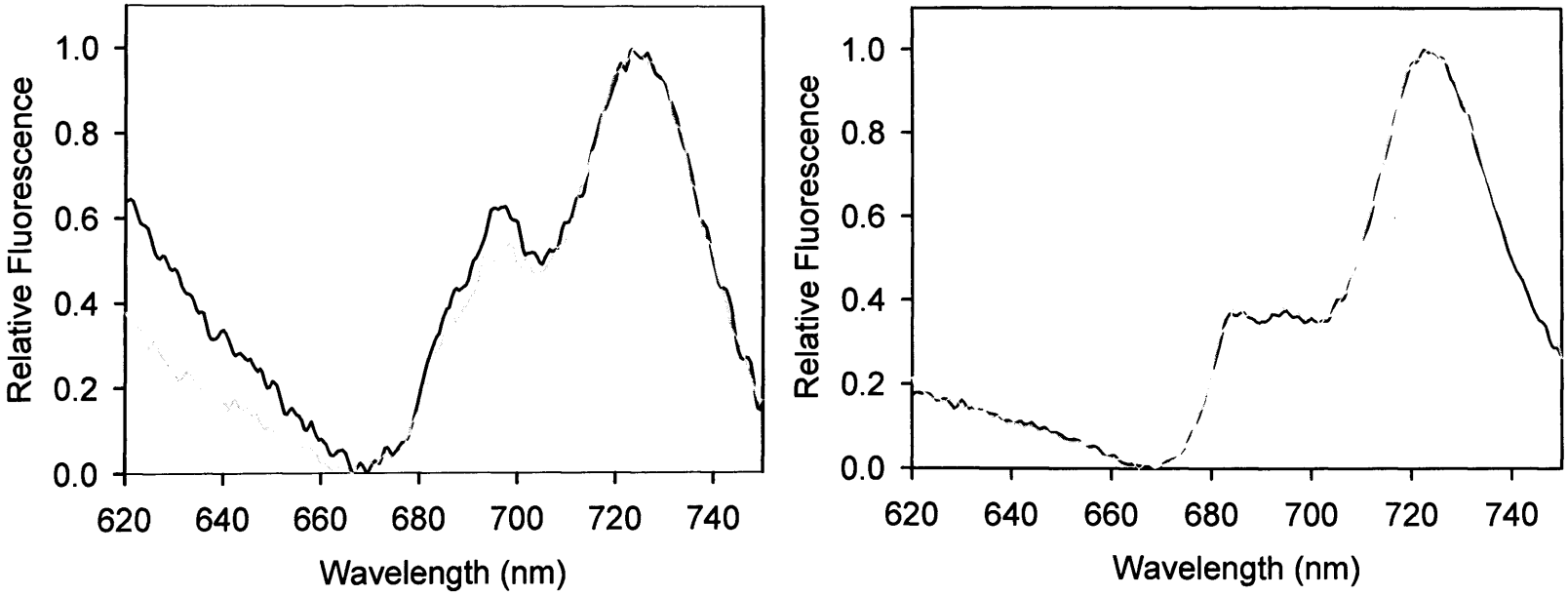


Figure 7.10. 77K whole cell fluorescence emission spectra for excitation of chlorophyll at 435nm of light (black) or dark (grey) adapted *Synechocystis* 6803 wild type (1) and *psb*28^{-} (2). Spectra were obtained using excitation and emission slit widths of 5nm, and were normalised to chlorophyll a fluorescence at 720nm.

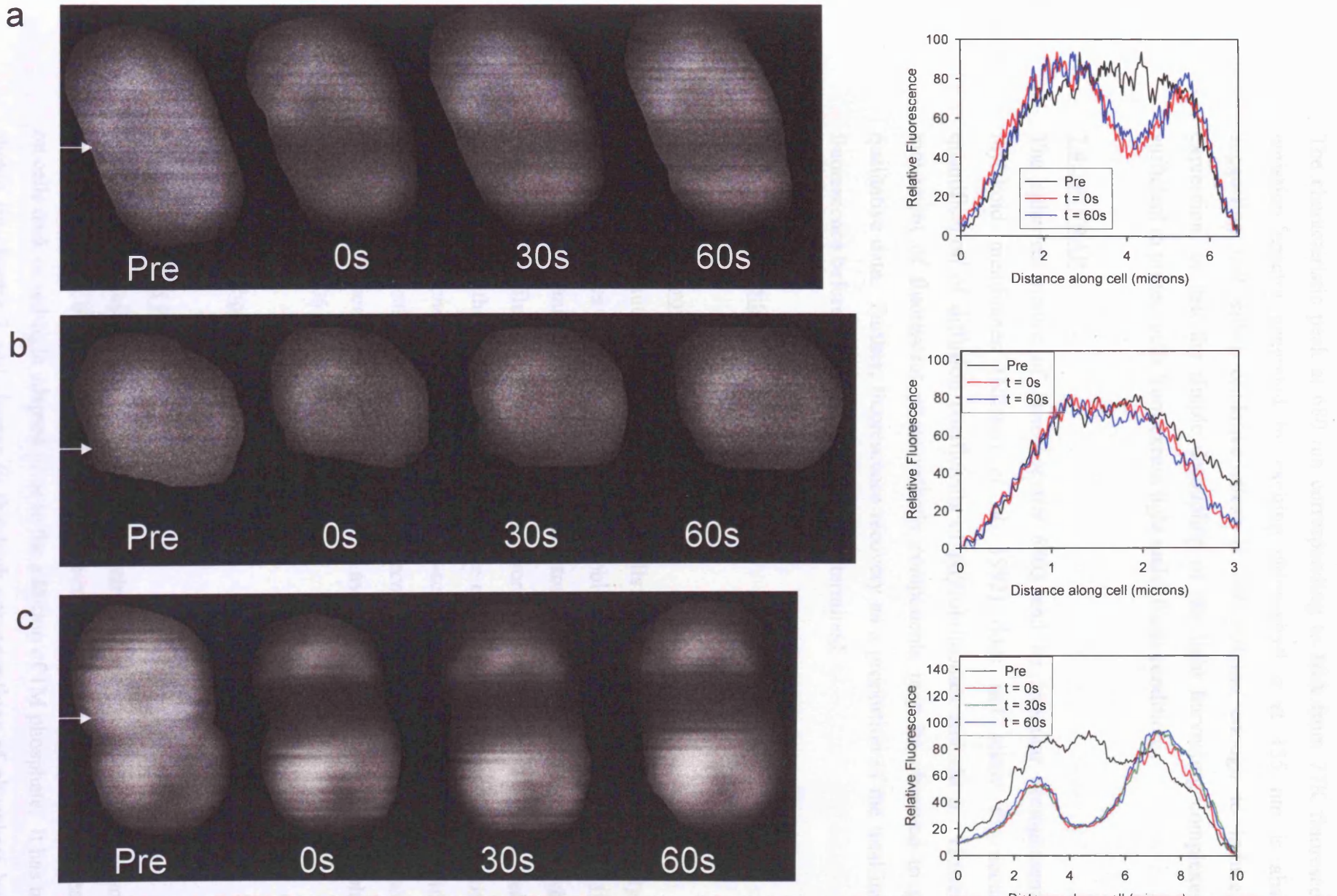


Figure 7.11. Chlorophyll FRAP of (a) *Synechocystis* 6803 WT, (b) *rpaC*$^{-++}$ and (c) *rpaC*$^{-}$ cells. Chlorophyll was excited at 457nm with an argon laser and fluorescence detected above 680nm. The highest intensity of bleach is in line with position of the arrow. Fluorescence profiles are given to the right of each cell presented, with the appropriate time-points.

The characteristic peak at 680 nm corresponding to IsiA from 77K fluorescence emission spectra generated by exciting chlorophyll *a* at 435 nm is absent, suggesting that either oxidative stress is not extreme enough to induce its expression, or that the simple decoupling of the light harvesting complexes is sufficient to protect cells from excess light under these conditions.

7.4.2.6. FRAP

The spherical nature of *Synechocystis* 6803 and its irregular arrangement of thylakoid membranes (Nilsson *et al*, 1992) does not allow for accurate quantification of diffusion coefficients of phycobilisomes and chl *a*. However, movement of fluorescent photosynthetic components may be observed to give qualitative data. Further, fluorescence recovery as a proportion of the total initial fluorescence before the bleach may also be determined.

7.4.2.6.1. Phycobilisome diffusion

7.4.2.6.1.1. On growth medium

Wild type and mutant *Synechocystis* 6803 cells display phycobilisome mobility on BG11 agar plates (data not shown). Phycobilisomes are mobile in the sll1399 inactivation mutant. It appears that phycobilisomes are moving faster in the *psb*28$^-$ mutant cells as fluorescence begins to recover even during the 2 second bleach, since the line of the bleach is blurred from the outset, but this cannot be quantified properly in *Synechocystis* 6803, on these time-scales. For this reason, it was critical to carry out the same experiment on *Synechococcus* 7942. In this species, *psb*28$^-$ diffusion coefficients are highly variable, but average out at a value comparable to the wild type (5.05 ($\pm$ 3.88) x 10^{-10} cm^2s^{-1}).

7.4.2.6.1.2. On phosphate

7.4.2.6.1.2.1. Red light fixed

Since the more rapid recovery in *Synechocystis* 6803 may be attributable to some de-coupled phycobilisomes increasing the rate of diffusion, the FRAP was repeated on cells dark or red-light adapted prior to the addition of 1M phosphate. It has been shown (in chapter 3 and chapter 6) that high concentrations of phosphate buffer

arrest state transitions by stabilising the binding of phycobilisomes which were associating with a particular reaction centre. However, if phycobilisomes are free, and un-coupled from reaction centres, the phosphate is unable to exert this kind of retardation. Fluorescence quenching and de-coupling of phycobilisomes suggested in the room temperature time-course and 77K fluorescence emission spectra respectively upon prolonged exposure to red illumination correlates with FRAP data which suggest partial fluorescence recovery in red-light adapted cells in the *psb*28$^-$ mutant only.

7.4.2.6.1.2.2. Dark fixed

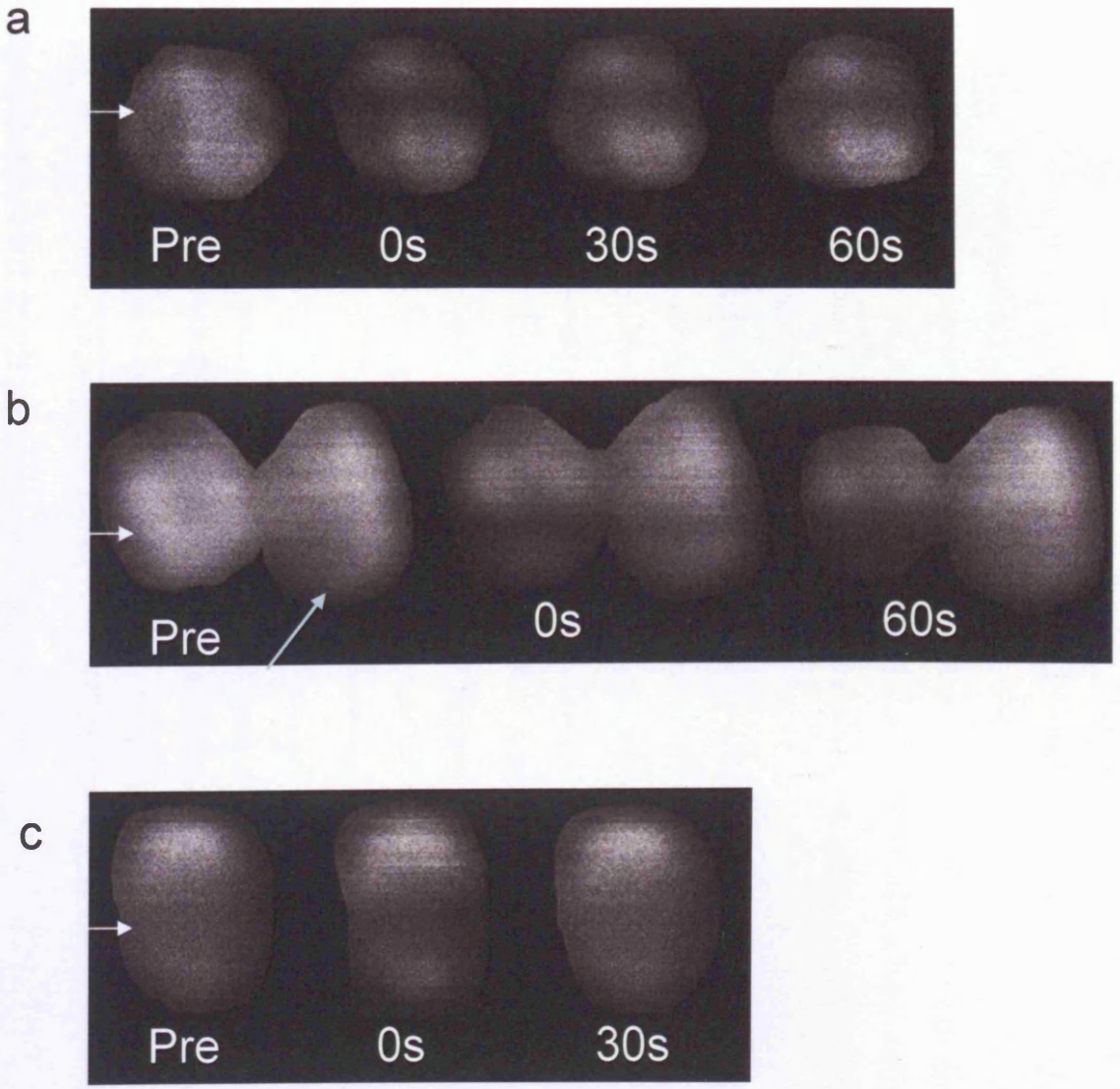
Wild type and mutant *Synechocystis* 6803 cells show no phycobilisome diffusion on the same time scale (30s) if pre-adapted to state 2 (in the dark) before the addition of the osmoticum (data not shown). The diffusion coefficient in 0.5 M sucrose for dark adapted *Synechococcus* 7942 *psb*28$^-$ is, at 3.45(+/-0.51) x 10^{-11} $cm^2.s^{-1}$ not significantly different from the wild type, though the light-fixed cells do appear faster (5.95(±4.03) x $10^{-11}cm^2.s^{-1}$).

7.4.2.6.2. Chlorophyll *a* diffusion

FRAP experiments undertaken using a 457nm Argon laser to bleach chlorophyll *a* show that PSII of mutant cells grown under moderate illumination conditions are able to diffuse. PSII from wild type *Synechocystis* 6803 is not capable of diffusion, this observation being consistent with observations with PSII in *Synechococcus* 7942 (Sarcina *et al*, 2001). Figure 7.11 shows (a) wild type and (b) *Synechocystis* 6803 GT *rpa*C^{-++} cells, with the fluorescence profiles provided adjacent to them. Whilst the control strains do not show chlorophyll diffusion, *psb*28$^-$ cells show variation in chlorophyll diffusion, ranging from no recovery to complete recovery. Figure 7.12 shows *Synechocystis* 6803 *psb*28$^-$ cells demonstrating this. The PSII diffusion coefficient for *Synechococcus* 7942 *psb*28$^-$ is 2.32 (± 1.51) x 10^{-11} cm^2s^{-1}, and recovery is partial, but varies from cell to cell.

7.4.2.7. Membrane preparations of state-1 or state-2-arrested suspensions

Membranes fixed in state 1 or state 2 were were extracted from both wild type and mutant suspensions using the high osmotic strength buffer SPCM (pH 6.8) (Gantt 1988). It was previously shown in chapter 3 that there is an increased amount of



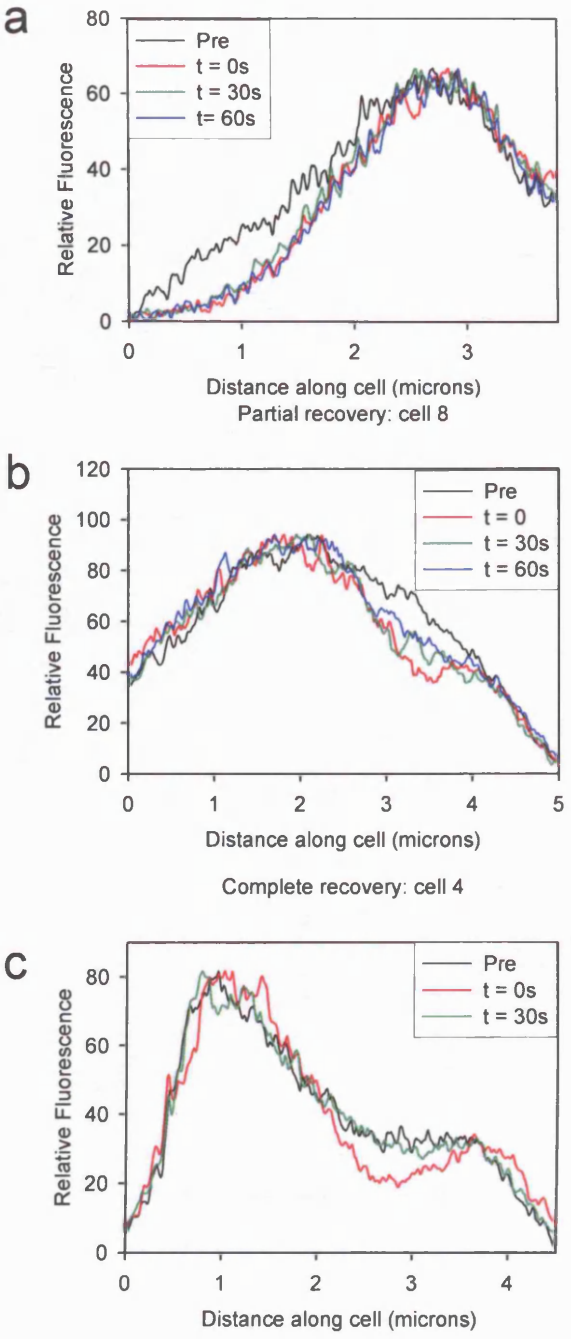


Figure 7.12. Chlorophyll FRAP of *Synechocystis* 6803 *psb*28^{-} showing (a) No recovery, (b) Partial recovery and (c) Complete recovery. Chlorophyll was excited at 457nm with an argon laser, and fluorescence detected above 680nm. Blue arrow indicates the cell with given fluorescence profiles for relevant time-points. White arrows indicate line where bleaching is at its most intense.

de-coupled phycobilisomes in state-2-arrested preparations, consistent with other observations from chapter 3, which show that phycobilisomes bind less strongly with PSI than PSII (Joshua and Mullineaux, 2004). Given that phycobilisome-PSII coupling appears to be in some way defective in *Synechocystis* 6803 *psb*28$^-$ cells, this experiment was carried out in order to determine if insertional inactivation of *psb*28 decreased or increased the affinity of the phycobilisomes for PSII. No consistent differences were observed.

7.5. Discussion

7.5.1. PSII diffusion

FRAP experiments to trace the movements of PSII chlorophyll in *Synechocystis* 6803 *psb*28$^-$ give a clear indication that PSII is mobile. The variable extent of diffusion may be explained by the fact that *Synechocystis* 6803 thylakoid membranes are irregularly arranged in the cell, and bleaching out of chlorophyll in a particular region could result in no recovery if that part of the cell loses access to other thylakoids outside the bleached region. PSII from wild type *Synechocystis* 6803 cells were observed to be immobile, consistent with FRAP data from other cyanobacteria (Mullineaux *et al,* 1997; Sarcina *et al*, 2001).

It has been demonstrated in *Synechococcus* 7942 that PSII diffuses when cells have been exposed to photo-inhibitory conditions prompting damage to the D1 protein and the induction of the PSII repair cycle (Mary Sarcina and Conrad Mullineaux, personal communication). In order to determine whether diffusion of PSII in *psb*28$^-$ is as a result of oxidative stress, another mutant (characterised in chapter 6 of this thesis) was used as a control. Since this mutant, *Synechocystis* 6803 *rpa*C^{-++} which shows defective light harvesting capability, is intolerant of high light and shows much lower fitness than the wild type also has PSII incapable of diffusion, it may be proposed that PSII diffusion in the case of *psb*28$^-$ is not simply an effect of oxidative stress. The Psb28 mutant shows chlorophyll diffusion regardless of whether grown under dim, normal or high light.

In cyanobacteria, PSII complexes are arranged in regular rows of dimers as shown by freeze-fracture electron microscopy (Mörschel and Schatz, 1987; Olive *et al*, 1997). A disorganisation of these rows would result in more PSII to PSI contact

(Mullineaux, 1999). Psb28 may be involved in maintaining the row-like conformation, in some way increasing the ability for phycobilisomes to couple to the dimers and to de-couple under the appropriate illumination conditions. Both increased affinity of the phycobilisomes with the reaction centre and the converse decreased affinity could affect efficient light harvesting. Increased affinity for PSII could increase the chance of photo-damage. Both factors could explain why phycobilisomes appear to de-couple from PSII following the transition to state 1 induced under red illumination conditions. An increased affinity for PSII in the mutant may explain why the state 1 transition is consistently faster than in the wild type, happening within a minute rather than over a 2-5 minute period. As the transition to state 2 is comparable to the wild type, it is unlikely that the de-coupling from PSII and subsequent re-association with PSI is inhibited by the absence of Psb28.

State transitions may be observed using 77K fluorescence emission spectra. It has previously been proposed that the observed increased fluorescence from PSII relative to PSI when adapted to state 1 as seen when exciting phycobilisomes is distinct from that observed when exciting the chlorophylls in the cells at 435 nm. While the mutant cells appear capable of state transitions as evidenced by tracing the phycobilisome-reaction centre interaction, the chlorophyll effect is not seen. State transitions were thought to be owing to excitation energy spilling over from chlorophylls in PSII to those in PSI. Since it is demonstrable that PSII of the mutant cells is mobile, it is feasible that reaction centres in disarray increase this spill-over.

7.5.2. PBS diffusion

Phycobilisome diffusion in the mutant occurs over a similar time scale to the wild type. In both wild type and mutant cells, the diffusion of the light harvesting complexes is complete within 30 s and is not dependent upon where in the cell the bleach is at its most intense. The lack of recovery of chlorophyll fluorescence in some mutant cells could be attributed to the absence of membranes spanning from one side of the cell to the other. Since phycobilisomes are not embedded in the membrane, this is not an issue, so recovery is expected regardless of the location of the bleach.

Previously, high osmotic strength buffers were used in order to determine the extent of fixation of state transitions in *Synechococcus* 7942, and to establish a link between the light-adaptation phenomenon and the diffusion of phycobilisomes (chapter 3 of this thesis and Joshua and Mullineaux, 2004). The results were not species specific, as fixation was also demonstrated in *Synechocystis* 6803. It has also been shown using the same technique that free phycobilisome diffusion onto IsiA complexes in the thylakoid could explain non-photochemical quenching in iron-starved *Synechocystis* PCC 6803 cells (chapter 6 of this thesis and Joshua *et al*, 2005). Since quenching is observed in *Synechocystis* 6803 psb28$^-$, fixing a state 1 transition is more complicated than in the wild type, but an investigation into phycobilisome diffusion of 1M phosphate-treated cells was carried out.

Unsurprisingly, the data presented show that in state 2-fixed wild type and mutant cells the phycobilisomes are immobile. This is consistent with there being no effect of Psb28 on the affinity of phycobilisomes for PSI and with the proposal that its absence does not inhibit the ability of the light harvesting complexes from de-coupling from PSII.

Differences were observed in phosphate-fixed cells which had been red-light adapted previously. In wild type *Synechococcus* 7942, state 1-fixed cells show slower phycobilisome diffusion than state 2-arrested cells, suggesting that phycobilisomes couple more strongly to PSII than PSI. In this study, state 1-fixed mutant cells show a small amount of fluorescence recovery from phycobilisomes, a property different from the non-recovering wild type cells. The recovery in the mutant cells could be as a result of phycobilisomes de-coupling from the reaction centre before the phosphate could completely fix them. Thus they would remain mobile owing to being free on the membrane. Another possibility would be that the absence of Psb28 decreases the affinity of the phycobilisomes for PSII. Both hypotheses may apply, but the latter is less likely given the observation that the mutant's transition to state 2 does not appear to be noticeably faster than in the wild type.

7.5.3. Fluorescence quenching following transition to state 1

Room temperature time-course measurements showing how phycobilisomes are associating with PSII show that while *Synechocystis* 6803 *psb*28$^-$ is capable of carrying out state transitions, the quenching of fluorescence is beginning approximately one minute after the red-light illumination treatment is started. This may be indicative of cells under oxidative stress reacting to excess illumination by channelling that excess from elsewhere following a de-coupling of the phycobilisomes. This is supported by red-light adapted and phosphate-fixed mutant cells, which show fluorescence recovery after photo-bleaching. The de-coupling of the phycobilisomes from PSII is also evidenced by 77K fluorescence emission spectra of mutant cells exposed to 3 minutes of red light prior to freezing. Red-light treatment of the cells does not appear to damage the mutant cells over short time scales, as they can be dark-adapted subsequently and then frozen, appearing no different from cells dark-adapted and frozen.

De-coupling of phycobilisomes from PSII and the diffusion of PSII may be linked. It is not clear whether the dissociation of the phycobilisomes is as a direct result of PSII diffusion and a potential disorganisation of the rows of dimers or whether the de-coupling of the light harvesting complexes prompts the chlorophyll diffusion. The former seems more plausible. A growth experiment of cells grown in blue light investigates whether light harvesting without the assistance of the phycobilisomes is significantly different in the mutant than the wild type. No significant difference in doubling times would suggest that the phycobilisome-de-coupling is the key to the PSII-diffusion phenotype. However, there is a difference, with wild type *Synechocystis* 6803 giving a doubling time of 71.67±1.53 hours compared with 114.33±3.06 for *Synechocystis* 6803 *psb*28$^-$ ($t = 21.64$, $p<2.7 \times 10^{-5}$). Therefore, the phycobilisome-dissociation may be deemed to be a secondary effect to the chlorophyll diffusion. If the spill-over of excitation energy from PSII to PSI normally applies to balance light harvesting to the reaction centres, inhibition of this might lead to excess energy transfer to PSII, prompting the de-coupling of phycobilisomes from PSII to quench the excess.

State transitions do not appear more convincing if cells have been grown under dim illumination conditions. However, the mutant does state transitions under high light, while the wild type does not. This indicates a possible role in

photoprotection, a theory supported by various groups (Rouag and Dominy, 1994, for example). This is not consistent with an alternative theory, which suggests that the role of state transitions is to maximise light harvesting under reduced illumination conditions (Murata, 1969 and Emlyn-Jones *et al*, 1999, for example). However, since it has been observed that light harvesting is impeded in this mutant, it may be that state transitions continue to be observed as a response to this, thereby maximising light harvesting.

7.5.4. Growth in dim light

The mutant appears to be at more of a disadvantage under dim illumination conditions than the wild type. This is indicative of a defect in light harvesting. It is feasible that the absence of Psb28 and a reorganisation of the morphology of the cyanobacterial thylakoid membrane may result in improved association of the light harvesting complexes with PSII (consistent with a fast transition to state 1). If this alters the balance in energy transfer to the two reaction centres, some signal could bring about the NPQ phenotype. Further, since the mutant has less chlorophyll per cell and typically less PSI molecules for a given amount of chlorophyll than the wild type, an increase in energy transfer to PSII would be predicted. Increased oxygen evolution per chlorophyll in the mutant compared with the wild type is consistent with this.

Since psb28 in *Synechocystis* 6803 is located within an operon, it is possible that its inactivation results in a combination of phenotypes arising from a down-regulation in expression of other genes within the operon. To exclude this, the knock-out construct of a gene immediately downstream was made (*Synechocystis* 6803-Δsll1399). This mutant gives a normal state transition phenotype and under moderate illumination conditions and PSII diffusion is not apparent.

As quantitative estimation of diffusion coefficients cannot be achieved in *Synechocystis* 6803 owing to its shape and irregular thylakoid arrangement, an inactivation of *psb*28 was made in the elongated cyanobacterium *Synechococcus* 7942. The gene is not present in an operon, so any phenotype may be more confidently attributed to the inactivation of solely this gene. The phenotype of this

knock-out mutant is consistent with that observed in the *Synechocystis* 6803 mutant, and PSII was observed to move, albeit partially.

Chapter 8: General Discussion

Chapter 8: General Discussion

8.1. Summary

Effective interactions of cyanobacterial light harvesting complexes with components of the thylakoid membranes are critical to establishing optimal photosynthesis given external environmental conditions without causing damage and the accumulation of toxins.

This body of work shows that phycobilisomes must be able to move rapidly on the thylakoid membrane and access both reaction centres. They are required to interact with both PSI and PSII, and it is important that the associations are transient. Binding too strongly or weakly could result in sub-optimal light harvesting and/or photo-damage.

The data presented in this thesis specifically provide information regarding:

- The absence of phycobilisome mobility and the corresponding effect on state transitions, a rapid light adaptation process
- The requirement for phycobilisome mobility for the quenching of excess light energy in iron starved cells
- A PBS-PSII binding component, RpaC, and the impact its absence/presence in two cyanobacterial species has on light harvesting and energy transfer
- An extrinsic PSII subunit, Psb28, conserved across all sequenced photoautotrophs, which appears to be required for maintaining organisation of the thylakoid membrane, specifically PSII anchorage. The effect on the regulation of light harvesting and reaction centre-reaction centre energy transfer when it is absent is examined.

Much of the data presented in the thesis rely on the use of high osmotic strength buffers. It has previously been shown that they are useful for locking cells in state 1 or state 2 (Mullineaux, 1994), but it is demonstrated here that the use of this method permits the observation of differential phycobilisome diffusion in alternately

adapted cells (Joshua and Mullineaux, 2004), as well as permitting the determination of the presence of de-coupled phycobilisomes (Joshua *et al*, 2005). The method has proved valuable for determining whether the presence or absence of a particular molecule assists or hampers PBS-reaction centre binding (Joshua and Mullineaux, *in press*). Data presented in chapter 4 of this thesis suggest that at this stage, the topology of the cyanobacterial thylakoid membrane (where phycobilisomes remain coupled to the reaction centres) cannot be elucidated via atomic force microscopy.

8.2. Introduction

Efficient photosynthetic capability by photoautotrophic organisms requires that cells are able to harvest light and transfer it to the photosynthetic reaction centres, PSI and PSII thereby making most effective use of the wavelengths available and causing minimal damage to these centres.

Central to the ability to do this, the light harvesting complexes must be capable of binding to the reaction centres, and detaching when appropriate. In cyanobacteria and eukaryotes, there is a differential need for transience in these interactions, as the reaction centre organisation is different in the thylakoids across this diverse array of phototrophic species.

The intensity of the interactions may be increased or decreased via covalent, conformational modifications to either the reaction centres or the light harvesting complexes, and these modifications may arise by virtue of complex signal transduction processes. Study of how these processes bring about such changes has been the focus of much research in the past twenty years or so. There is, however, much that remains elusive to the photosynthesis research community, particularly with regard to the extent to which such processes are conserved between photosynthetic prokaryotes and their eukaryotic relatives.

What follows is a discussion of how optimal binding may be achieved, and how pigment-protein complexes may be used to protect the organism. The focus of this

thesis is cyanobacteria, but a comparison of the mechanisms in the wider context of photosynthetic eukaryotes is also discussed.

8.3. Phycobilisome binding to reaction centres and IsiA

8.3.1. Light harvesting

Cyanobacteria are capable of efficient light harvesting to both PSII and PSI via phycobilisomes as well as chlorophyll. Light harvesting by chlorophyll in PSI is particularly efficient, but PSII relies more strongly on receipt of energy from phycobilisomes. Given this, it would be unsurprising if an alteration of the phycobilisome-PSII interactions resulted in cells presenting a detrimental phenotype.

It has been demonstrated using high osmotic strength buffers that it is possible to arrest cells in state 1 or state 2 (Mullineaux, 1993; Li *et al*, 2004; Joshua and Mullineaux, 2004). More phycobilisomes are interacting with PSII in state 1 than in state 2, and that there are more phycobilisomes interacting with PSI in state 2 than in state 1. It is not known precisely how the buffer locks the phycobilisomes onto the reaction centres, but it is possible that a low A_w stabilises the protein complexes.

The initial aims of the project described in this thesis were to determine what triggered a differential association of phycobilisomes with thylakoid complexes. It was hoped that this would help to determine how the phycobilisomes associated with these pigment-protein complexes. In chapter 3 (and Joshua and Mullineaux, 2004) it was established that in both *Synechococcus* 7942 and *Synechocystis* 6803 the interaction between the light harvesting complexes and PSII was stronger than that of PSI in healthy wild type cells. Work presented in chapters 4 and 5 showed that RpaC, a membrane-targeted protein conserved in cyanobacteria, alters the strength of phycobilisome-PSII complexes, and results in cells showing adverse phenotypes.

RpaC was previously eliminated in *Synechocystis* 6803 giving a very specific state transition negative phenotype. It was therefore proposed that RpaC was specifically required for state transitions, and that it was likely to be some kind of signalling

molecule (Emlyn-Jones *et al*, 1999). However, studies of an RpaC knock-down mutant in *Synechococcus* 7942 (which remained state transition competent) suggested otherwise. Using certain osmotica, namely phosphate buffers or sucrose solutions, it can be shown that in this mutant, the phycobilisomes interact less strongly with PSII, as determined by the phycobilisome diffusion coefficients being larger for state 1-fixed mutants than wild type cells exposed to similar pre-fixation light adaptation conditions. It is possible that this observation is of physiological importance if altering the intensity of the PBS-PSII interaction has a massive impact on the photosynthetic capability and well-being of the cells.

It is likely, given the data presented in chapters 4 and 5, that RpaC is a PBS-PSII binding factor which has an impact on the flexibility of light harvesting to the reaction centres, and therefore in conditions where it is present in larger/smaller amounts, can indirectly prevent or enhance the appearance of state transitions.

Insertional inactivation of the gene in *Synechococcus* 7942 does not prevent the ability to carry out state transitions, so it may be that it is not a state transition requirement in this species. This should be taken with caution, as the mutant would not segregate. However, if complete inactivation of *rpa*C is lethal to the cells on account of the cells being incapable of state transitions, other mutants incapable of state transitions would not be predicted to survive either. This is not the case, as *Synechocystis* 6803 *rpa*C$^-$ performs almost as well as the wild type (Emlyn-Jones *et al*, 1999). Further, there are other cyanobacterial mutants which also show a state transition negative phenotype and are fully segregated, for example, the inactivation of the *apc*D gene of *Synechococcus* 7002 which encodes part of the phycobilisome core (Zhao *et al*, 1992). It would be interesting to determine, via the use of phosphate buffers, whether the ability to associate or decouple the phycobilisomes from the reaction centres is impeded in phycobilisome core mutants. This might give a better understanding regarding whether the state transition "signal" is more or less important than the phycobilisome-reaction centre coupling properties.

The fact that the *rpa*C mutant in *Synechococcus* 7942 is still capable of state transitions might be because differential PBS-PSII binding does not impair the redox signalling in a transduction cascade which may be necessary to catalyse state

transitions. If the mutant is more state 2-like, there may be another signal which tells the cells to balance things out by shifting cells towards state 1 where appropriate.

The non-segregation of the mutant in *Synechococcus* 7942 may be owing to the differential PBS-PSII binding, specifically the observation that the binding is weaker in the mutant. These cyanobacteria, when arrested in state 1, had phycobilisome diffusion coefficients significantly larger than those in similarly treated wild type cells. There is no difference between state 2 fixed mutant cells and those of the wild type, suggesting that the strength of PBS-PSI interactions is not affected by the reduction of RpaC in the membrane. That state 2 fixation estimates are higher for the mutant than the wild type may simply be on account of the PBS-PSII interactions being weaker in the mutant, which therefore make it more likely that a phycobilisome becomes fixed to a PSI reaction centre.

Mutant cells respond to the difficulties in light harvesting by adjusting pigment content. The up-regulation of PSII synthesis in both *Synechococcus* 7942 *rpa*C$^-$ and the *Synechocystis* 6803 over-expression mutants is likely to be a secondary effect caused by reduced efficiency of light harvesting to PSII reaction centres. Mutants with reduced light harvesting by phycobilisomes to PSII, for example those without phycobilisomes, (Tetenkin *et al*, 1997; Ajlani and Vernotte, 1998, for example) or small phycobilisomes (Zhao *et al*, 1992) up-regulate the amount of PSII that is synthesised, probably in response to some redox signal.

PSII damage often occurs when there is excessive light harvesting through this reaction centre, as the D1 polypeptide encounters oxidative damage by free radicals (see Keren and Ohad, 1998, for review). If we expect that RpaC strengthens PBS-PSII interactions, up-regulating RpaC (as seen in the *Synechocystis* 6803 mutants described in chapter 5) might increase this still further. Damage to the reaction centres would prompt a response in the cells with respect to the synthesis of more PSII reaction centres (see PSII/PSI ratios and quantities). Increased PSII synthesis is seen, but this would also be expected if PBS-PSII is not strengthened, which is in fact what was observed in these over-expression mutants. Reducing the strength of PBS-PSII coupling in the *rpa*C mutant of *Synechococcus* 7942, may indicate to the

cells that less energy is being transferred to PSII, so they up-regulate it to compensate. It has previously been reported that state transitions have a photoprotective role. A study in *Synechococcus* 6301, a bacterium near identical to *Synechococcus* 7942, reported that under conditions where damage to D1 was likely to occur, a transition to state 2 would occur, to divert some of the excess light energy away from PSII and towards PSI instead (Rouag and Dominy, 1994). If this is so, it may explain why the mutant in *Synechococcus* 7942 remains state transition competent, even when RpaC is downregulated.

Another, perhaps more outlandish possibility may also be considered. Perhaps "less strong" interactions actually correspond to more "efficient" ones? Given that high osmotic strength buffers increase the interaction but reduce the amount of energy transfer, it is not unreasonable to suppose that RpaC could exert stronger physical interactions by virtue of an alternate conformation are detrimental and cause damage. It is feasible that in order to permit flexibility in light harvesting, cyanobacteria have evolved to have a protein which gets in the way of a more intense binding, but actually results in a less extreme transfer of energy. RpaC may have evolved to be intrinsic to protecting PSII from damage caused by excessive transfer by altering the conformation of a PBS-PSII complex. This may explain why it is conserved in cyanobacteria.

If the presence of RpaC results in stronger PBS-PSII interactions, it is surprising that *Synechocystis* 6803 *rpaC*$^-$ appears to be arrested in state-1. The fact that the *Synechocystis* 6803 *rpaC* deletion mutants are not at as much of a disadvantage as the corresponding mutant in *Synechococcus* 7942 may be owing to the different number of phycobilisome core cylinders – such a structural difference may mean that a slightly weaker phycobilisome-PSII interactions are less of a problem for some reason. Both *rpaC* mutants (the knock-down in chapter 4 and the over-expressor in chapter 5) are slow growing in dim light because of imbalances in light harvesting, either caused by the distorted PSII/PSI ratios, or, more likely, less efficient energy transfer to PSII.

The high osmotic strength buffers used to investigate phycobilisome mobility and state transition fixation in the *Synechococcus* 7942 *rpaC*$^-$ mutant suggest that RpaC

is more likely to be a binding molecule than a signalling molecule. Over-expression of RpaC may have been predicted to give a state transition positive phenotype under all growth conditions if RpaC was involved in signalling. In fact, placing *rpa*C downstream of the *psb*A2 promoter in an *rpa*C$^-$ background did not even rescue a state transition phenotype. From this we can propose that up-regulating RpaC clutters the already crowded thylakoid membrane and in some way interferes with light harvesting. If, as proposed in chapter 5, free RpaC collects in the thylakoids and phycobilisomes bind to it upon changing the illumination conditions to those which favour the state 1 transition (that is, red wavelengths), quenching of fluorescence as seen in the room temperature spectra could be explained. That said, we cannot discount the possibility that RpaC is involved in both binding and signalling.

Both over-expression mutants are very slow growing under all illumination conditions tested. This may be attributable to reaction centre damage, particularly PSII, where it appears that cells have responded by synthesising IsiA in high light and inducing NPQ. Carotenoid levels are massively increased in these mutants and this in itself points to the cells being under severe oxidative stress (Silva *et al*, 2003, for example). The NPQ observed may not be on account of IsiA, and may be as a result of phycobilisomes binding to excess RpaC in the membrane, as previously discussed.

In contrast with the phycobilin-possessing cyanobacteria, the light harvesting complexes in green plants, LHCI and LHCII contain both chlorophyll *a* and chlorophyll *b*. Their reaction centres possess solely chlorophyll *a* (Peter and Thornber, 1991) and each LHC has a tendency to associate with just one of the reaction centre complexes, PSI or PSII respectively. Far more is known about the reaction centre subunits involved in interaction with the LHCs in plants (Zhang and Scheller, 2004, for example) than is known about the interactions of phycobilisomes with PSI and PSII in cyanobacteria.

LHCII is comprised of three functionally distinct types, present in multiple copies and conserved across plant species, LHCB1, 2 and 3. These types are, to differing extents, involved in maintaining flexibility in light harvesting. Lhcb2, in

Arabidopsis thaliana, at least, is the most abundant, and is apparently involved in both short and longer term light adaptation. This is owing to its presence in the periphery of LHCII, where, due to an up-regulation under low light conditions, the antenna enlarging, hence increasing the ability to harvest light under these suboptimal conditions (Larsson and Andersson, 1985; Larsson *et al*, 1987). While Lhcb1 also contains a phosphorylation domain, faster phosphorylation kinetics have been recorded for Lhcb2, and it is therefore likely that the rapid term light adaptation phenomenon of the state transition is owing to the reversible phosphorylation of this particular Lhcb protein. LHCII is also involved in photoprotection, and the observation of NPQ requires its presence.

Effective binding of LHCI to PSI requires the presence of some key PSI subunits. It has been observed in pea plants that there is decreased stability of the Lcha peripheral antenna with PSI in the absence of PsaG (Jensen *et al*, 2002). In *Arabidopsis*, it is known that PsaL and PsaH bind 5 chlorophylls, which are probably involved in energy transfer from LHCII to PSI (state 2) (Ihalainen *et al*, 2002). In cyanobacteria, PsaL$^-$ mutants cannot form trimers, and have faster transitions from state 2 to state 1 (Aspinwall *et al*, 2004).

Whilst cyanobacteria have phycobilisomes which bind to both reaction centres, higher plants have LHCII and LHCI. LHCI is membrane bound, and binds to PSI. In most plants, the antenna comprises four subunit polypeptides (Lhca1-4), each of which binds 10 Chl *a* or *b* molecules and some xanthophylls. It is likely that they are involved in both light harvesting and photoprotection. LHCI binds to the PSI core in such a manner that it would not inhibit the ability for PSI to trimerise, though PSI in plants is monomeric (Boekema *et al*, 2001). The highly efficient energy transfer between this LHC and PSI is not in question. Unicellular photosynthetic eukaryotes like *C. reinhardtii* have larger PSI-LHCI complexes than their multicellular relatives (Germano *et al*, 2002). Some have proposed that the size difference is somehow related to the requirement of cells to carry out state transitions. In cyanobacteria, the mere fact of phycobilisomes binding to PSI directly has been the subject of intense debate over the past twenty years, but there is now much evidence in favour of it (Mullineaux, 1992 and 1994; Rakhimberdieva *et al*, 2001).

Flexibility in light harvesting in plants is just as crucial as it is in photo-autotrophic prokaryotes. In *Arabidopsis thaliana*, mutants exist which show that tampering with pathways involved in the light state transitions or non-photochemical quenching of excess fluorescence give deleterious phenotypes (Peterson and Havir, 2001; Pesaresi *et al*, 2002; Bellafiore *et al*, 2005, for example). However, it was reported that the effect is quite subtle. That is, genes involved in Q_E may provide a selective advantage under field conditions where illumination conditions fluctuate, rather than just provide greater tolerance to high light (Kulheim *et al*, 2002).

The well understood state transition phenomenon in higher plants is dependent upon the reversible and light-activated phosphorylation of light harvesting complex II (LHCII) (Rintamaki *et al*, 1997; Hammer *et al*, 1997) by a kinase. This kinase is specifically targeted to subunits Lhcb1 and Lhcb2 within LHCII, and upon reduction of the plastoquinone pool, and the association of these molecules with the Q_o site of cytbf (Vener *et al*, 1997), phosphorylation of the Thr-5 residue occurs (Zer *et al*, 1999), which consequently brings about the detachment of the light harvesting complex from PSII. Zito *et al* (1999) determined that if the occupation of Q_o by plastoquinol is inhibited, the kinase will remain inactivated. The mutant characterised by Zito *et al* is a *pet*D mutant of *C. reinhardtii* which is incapable of state transitions and is perpetually in state 1. It is evident that the ability of this kinase to function is critical to the cells under certain conditions, since there is a certain amount of evidence that some phosphatase activity can still exist regardless of the wavelength of light cells are provided with, and irrespective of the redox state of the plastoquinone pool (Elich *et al*, 1997).

Cytbf. mutants of *C. reinhardtii* mutants cannot activate the kinase on account of the absence of a Q_o site. They therefore remain in state 1 permanently (Lemaire *et al*, 1986; Wollman and Lemaire, 1988; Gal *et al*, 1990). Gal *et al* (1988) have reported similar findings in a *Lemna* mutant lacking the Cytbf.

It has not been possible to isolate LHCII-PSII supercomplexes from *C. reinhardtii* membranes extracted from organisms grown under state 2 conditions. Those grown in state 1 do permit isolation of such supercomplexes. It is likely, therefore, that the

affinity of LHCII for PSII is much smaller in state 2 than in state 1, and is a factor additional to the prerequisite for phosphorylation of the LHCII for the observation of state 2 (Dekker and Boekema, 2005). In cyanobacteria, it is possibly the location of RpaC which alters the intensity of PSII-phycobilisome binding which permits state transitions to occur.

It is evident that the requirement for a conformational change to bring about state transitions is conserved throughout phototrophic species. It is debatable whether this is brought about via a phosphorylation event in cyanobacteria. In plants, upon phosphorylation of LHCII and the consequent detachment from PSII, some LHCII then forms an association with PSI (Zhang and Scheller, 2004, for example). In addition to the proposed LHCII binding site at PsaH of PSI (Lunde *et al*, 2000, Pesaresi *et al*, 2002), there has also been a suggestion that it can be induced upon the onset of senescence (Prakash *et al*, 2003). Spill-over from PSII to PSI is not well understood in plants or in cyanobacteria, but in the latter in particular, there is intense debate. The Psb28 mutant in *Synechocystis* 6803 (chapter 7) may have shed some light on the subject, in that a chlorophyll effect of state transitions is lost. This effect appears to be independent of the associations of the phycobilisomes, and is more likely to be on account of a rearrangement of thylakoid topography (see section 8.5.). It is interesting, however, that in the *Synechocystis* 6803 *psb*28 mutant, this likely disorganisation of thylakoid components results in phycobilisomes binding to PSII more quickly, but detaching in a manner quite unlike the wild type.

8.3.2. NPQ

Apart from light harvesting, the ability to prevent excess photo-damage to the reaction centres relies on the ability of certain molecules to access others and interact with them appropriately. IsiA is a chlorophyll-complex which has been implicated in light harvesting and cyanobacterial NPQ. It is not detected in higher plants, but owing to its presence in cyanobacterial PSI trimers, it has also been established that it significantly increases the efficiency of light harvesting to PSI under conditions of iron starvation (Melkozernov *et al*, 2003; Andrizhiyevskaya *et al*, 2004). It has been proposed that the formation of IsiA complexes in the thylakoid membrane of cyanobacteria is involved in the NPQ phenomenon. The

formation of such peripheral antenna complexes is therefore, not solely restricted to higher plants, since this is seen, albeit rarely, in primitive photoautotrophic organisms.

To explain cyanobacterial NPQ, and given the understanding that in iron-starved cells there is a proportion of free phycobilisomes, it was also proposed that these phycobilisomes remain freely mobile in phosphate buffer until they become associated with IsiA complexes in the thylakoids (chapter 6 and Joshua *et al*, 2005). While it is currently impossible to culture *Synechocystis* 6803 phycobilisome-less mutants or IsiA$^-$ mutants under conditions of prolonged iron starvation, to study NPQ, it may be possible to carry out studies of mutants with minor mutations of the allophycocyanin cores. This may permit a more thorough investigation of how phycobilisome-IsiA interaction may be required for the poorly understood NPQ phenomenon in cyanobacteria.

It is evident that the signalling and binding processes in plants which lead to NPQ are far better understood than in prokaryotes. Zeaxanthin has been confirmed to be the xanthophyll required for efficient quenching of excess excitation energy in plants. This was determined by generating mutants which would disrupt the violoxanthin cycle which brings about the enymatic conversion of violoxanthin to zeaxanthin, catalysed by a transmembrane pH gradient in conditions where light is in excess. The protonation of PsbS is also required (Li *et al*, 2004), which then, in turn, binds the zeaxanthin (Aspinall-O'Dea *et al*, 2002) and triggers fluorescence quenching. There are two, widely debated proposed mechanisms for how zeaxanthin brings about NPQ in plants. The first suggests that zeaxanthin exerts a purely structural effect (Demmig-Adams, 1990), and the other proposal relies on the possibility that it exerts an allosteric effect, where the quencher is a chlorophyll dimer formed during the conformational transition brought about by zeaxanthin binding (Horton *et al*, 2000).

8.4. Phycobilisome mobility

This is a primary requirement for permitting flexibility in light harvesting. State transitions require the phycobilisomes to be fast moving.

We have exploited the fact that high osmotic strength buffers act to stabilise phycobilisome-reaction centre complexes (Joshua and Mullineaux, 2004) to study a mechanism not previously known to require phycobilisomes to be mobile. Iron starved *Synechocystis* 6803 produce large quantities of IsiA (Boekema *et al*, 2001), some of which complexes to aid light harvesting to the reaction centres, in particular, PSI (Melkozernov *et al*, 2003; Andrizhiyevskaya *et al*, 2004). There is a sub-population of IsiA, produced in excess of that which is used in light harvesting, which complexes with themselves, remaining in the thylakoid membrane. NPQ has been observed in such iron stressed cyanobacteria, but not in cells grown in iron replete conditions. NPQ is common in plants, and is a process widely thought to be necessary to prevent photo-damage to the reaction centres under conditions of oxidative stress (see Holt *et al*, 2004 for review).

Aside from IsiA expression, iron-starved cyanobacteria differ from non-stressed cells in that a small proportion of their light harvesting complexes are free, and un-coupled to reaction centres (Joshua *et al*, 2005). Observing this, we postulated that upon addition of high osmotic strength buffer, free phycobilisomes would remain free, but those associating with membrane complexes (reaction centres or IsiA) would become fixed to those complexes. A high osmotic strength buffer (1 M phosphate, pH 6.8) did not prevent the induction of NPQ, but fluorescence recovery in the dark was strongly inhibited. We suggested that free phycobilisomes diffuse on the membrane and associate with IsiA to induce quenching, but once associated with the complex are then unable to detach in the presence of the buffer. FRAP experiments supported this hypothesis.

Now, with respect to plants, it has been commented upon (Dekker and Boekema, 2005) that if violoxanthin de-epoxidase is active at the inner surface of the thylakoid membrane, zeaxanthin would not be required to move long distances to the PSII-associated PsbS subunit to activate quenching. It is notable that PsbS does not associate with PSII when PSII is within an LHCII-PSII supercomplex (Nield *et al*, 2000), suggesting that for quenching of fluorescence to occur, LHCII must first detach via some signal, and then PsbS must attach, followed by the binding of zeaxanthin. In cyanobacteria, mobility of the free phycobilisomes is likely to be crucial to the observation of F_o quenching, while the F_v quenching appears to be

under the control of a different mechanism. We established this by showing that phosphate does not inhibit the induction of quenching (suggesting phycobilisomes which were not associating with the reaction centres at the time). Iron deprivation to a less extreme extent does not induce the capability for F_o, but does permit the observation of F_v. Since there do not appear to be a large proportion of decoupled phycobilisomes at this level of iron deprivation, and quenching is not inhibited by the phosphate, it is likely that F_v and F_o quenching are controlled by two different mechanisms (Joshua *et al*, 2005) and that the former does not require the mobility of free light harvesting complexes.

In *Chlamydomonas*, a state 2 transition requires that the LHCII moves from the grana to the stromal lamellae of the chloroplast (that is, from PSII to PSI domains) (Dekker and Boekema, 2005, for review). Further, a migration of the cytbf complex is required (Vallon *et al*, 1991), as is the up-regulation of cyclic electron flow around PSI (Finazzi *et al*, 2002). The proportion of LHCII involved in migration to bring about a state transition is significant, and variable from species to species. In *Chlamydomonas*, approximately 80% of the total is required to have migrated (Delosme *et al*, 1996), while in green plants, the requirement is far smaller, with only about 20% moving (Allen, 1992). Data presented in chapter 3 of this thesis confirms that in cyanobacteria, long range diffusion of the majority of light harvesting complexes is also a critical requirement for the observation of light state transitions (Joshua and Mullineaux, 2004). The variability between the species appears to correlate with the membrane stacking differences. Cyanobacteria do not have grana, and *Chlamydomonas* possesses far less stacking than that observed in higher plants (Olive *et al*, 1996). As such, phycobilisomes probably have to move less distance to achieve a state transition. LHCII in *Chlamydomonas* probably have greater access to the kinases required for the catalysis of the state 2 transition, so, the higher proportion that are mobile probably correlates with the greater quantity that become phosphorylated upon alteration of the illumination conditions.

8.5. The role of PSII immobilisation/oligomerisation

Organisation of PSII dimers has been shown to bear significant similarity between cyanobacteria and higher plants. This organisational conservation suggests an important functional role of dimeric aggregates of PSII (Mullineaux, 2005, in press). How the organisation is controlled is the subject of intense debate in both prokaryotic and eukaryotic photoautotrophs. In higher plants, it has been proposed that the aggregates of dimers are stabilised via protein phosphorylation or the binding of particular PSII subunits. In cyanobacteria, the membranes do not exhibit the regularly stacked property typical of eukaryotes (Mullineaux, 1999). The importance of grana in higher plants has also been the subject of discussion, and some groups have suggested that the stacking prevents the loss of excitation energy. The Psb28$^-$ cyanobacterial mutant generated (chapter 7) may shed light on this subject, in that the normally immobile PSII is seen to diffuse rapidly in this mutant, which also shows deficiencies in light harvesting. Psb28 is a conserved PSII subunit and this may play a critical role in the PSII organisation process. In *A. thaliana*, it has also been recorded that in order to observe LHCII-PSII supercomplexes, a dimeric PSII structure is imperative. One could suppose that in the absence of such rigid PSII organisation, light harvesting capability would decrease. We see this in cyanobacteria, where the *psb*28$^-$ mutants show defective light harvesting in white light, but also in blue light (non-phycobilin absorbed).

The total number of trimeric LHCII complexes for each PSII dimer has been approximated to about eight (Peter and Thornber, 1991). This is variable according to species, but also with respect to the composition of the LHCII complex itself. (For example, Lhcb3 is rarely found in the larger complexes.)

There has been intense debate by groups working on light state transitions in cyanobacteria as to the underlying mechanisms for the phenomenon (Allen and Forsberg, 2001, for review). Many still hold with the notion of there being a "spill-over" of excitation energy from PSII to PSI upon altering the illumination conditions to favour a transition to state 2. This was originally proposed by Murata (1969) working on the red alga *P. cruentum*. Freeze fracture electron microscopy has shown that PSII dimers are locked in rigid rows on the thylakoid membrane (Olive *et al*, 1986; Mörschel and Schatz, 1987; Nilsson *et al*, 1992). Olive *et al* (1986), working on *Synechocystis* sp PCC 6714, reported findings that a

rearrangement of the rigid rows of PSII dimers permitted a transition to state 2. The same principle of pre-adapting cells to state 1 or state 2 before freeze fracture studies of the membranes was applied to *Synechocystis* 6803, yielding similar results (Olive *et al*, 1997). They proposed, once again, that this facilitated a spill-over effect, and showed that in thylakoids of *Synechocystis* 6803 *ndh*B$^-$, a mutant incapable of state transitions (Schriber *et al*, 1995), randomisation of the PSII rows did not occur (Olive *et al*, 1997). Others, favouring a model reliant on the rapid movement of phycobilisomes between the reaction centres, show data consistent with this hypothesis instead (Mullineaux, 2001, for review).

77K fluorescence emission spectra show clear differences in the ratio of PSII/PSI fluorescence in the two light states when exciting chlorophyll (at 435 nm), but also when exciting phycobilisomes (at 600 nm) showing the relative amounts bound to reaction centres. Mullineaux (2004) proposes that these two effects are separate, and brought about by different mechanisms, citing the state transition-defective *Synechocystis* 6803 *rpa*C$^-$ mutant, which retains the chlorophyll effect by lacks the phycobilisome effect (Emlyn-Jones *et al*, 1999). Even wild type cells, when not carrying out the phycobilisome-related effect still show the chlorophyll phenotype. Further, a mutant of *Synechococcus* sp. PCC 7002 which lacks phycobilisomes still produces increased PSII fluorescence relative to PSI when cells have been adapted to state 1 (Bruce *et al*, 1989). Meunier *et al* (1997), working on the cyanobacterium *Cyanothece* sp. ATCC 51142, suggested that alterations in the oligomerisation of reaction centres may result in fluorescence changes upon alteration of the illumination conditions to promote one or the other state. The oligomerisation of PSI, notably in *Synechocystis* 6803 *psa*L$^-$, does not impair the chlorophyll effect though (Aspinwall *et al*, 2004), suggesting that the trimerisation of PSI is not critical to its observation either. The *psb*28$^-$ mutant in *Synechocystis* 6803 may shed light on the requirements for the latter effect, but it is important to note that the corresponding knock-out in *Synechococcus* 7942 gave a normal chlorophyll effect comparing spectra for red light and dark adapted frozen cells.

Synechocystis 6803 *psb*28$^-$ is capable of state transitions as judged by the phycobilisome fluorescence properties at 77K and at room temperature. The chlorophyll effect is not observed under any of the growth regimes used, but

interestingly, the normally immobile PSII is seen to diffuse rapidly (at a similar rate to the light harvesting complexes). PSII diffusion has previously only been observed when cells have been exposed to prolonged photo-inhibitory conditions, and fluorescence recovery in the bleached region from FRAP experiments in *Synechococcus* 7942 has only ever been deemed to be "partial" (Sarcina, personal communication). The oxidatively stressed *Synechocystis* 6803 *rpa*C^{-++} mutant has immobile PSII complexes, suggesting that the effect in the *psb*28^{-} mutant is not a simple stress effect. It is feasible that PSII mobility in the latter is the critical factor in preventing the observation of increased chlorophyll fluorescence from PSII compared with PSI following state 1 acclimation conditions. Perhaps it is also preventing the spill-over of excitation energy to PSI. This supports the freeze fracture data presented by Olive *et al* (1986) and the model described by Mullineaux (1999) which would require that disordering of PSII rows would lead to increased interactions between PSII and PSI, and therefore increase the potential for "spill-over", if indeed that was the mechanism for the chlorophyll effect. If the PSII are already disordered in state 1, a chlorophyll effect upon a transition to state 2 would not be observed.

Under blue light (that is, illumination wavelengths not absorbed by phycobilins) growth conditions the mutant is still at a disadvantage, suggesting that the absence of the chlorophyll effect is a problem with respect to the cells' light harvesting capabilities. Since the chlorophyll effect is generally seen in other species under all conditions published so far, supports the reasoning that the effect is an important requirement for the cells.

Now, we should be cautious in inextricably linking the chlorophyll effect with the observed diffusion of PSII, as the topography of the thylakoid membrane is likely to be quite different in the *psb*28^{-} mutant compared with the wild type on account of the massive down-regulation of PSI. This property in itself might be catalysing the mobility of PSII via some sort of signal. Further, it is possibly that simply reducing PSI quantity may account for the absence of the chlorophyll effect on account of there being fewer of these reaction centre complexes for the excess excitation energy in PSII to spill over to. An alternative way of interpreting what is happening might be that poor PSII organisation in the membranes of the mutant results in

thylakoid overcrowding, resulting in the cells responding by reducing the quantity of reaction centre(s).

The amount of membrane stacking is variable, but may be in the order of several dozen membranes in the vertical direction. Protrusions from the membranes are different on each side given the intra-membrane complexes. PsbP and PsbQ are the most exposed of the PSII subunits, and they contribute to the shape of the granal stacks. The vertical distance of grana was shown to be changed according to the illumination conditions (Albertsson, 1982), which may reflect the thylakoid organisation variability between state 1 and state 2 conditions. In cyanobacteria, Olive *et al* (1996) suggest that randomisation (that is, the loss of rows) of the thylakoids occurs upon the transition to state 2.

PSI and the ATP synthase are found in unstacked thylakoids owing to their large stromal protrusions. They are also located on the end of the grana. It has been demonstrated that chlorophyll *b* deficient mutants are incapable of producing normal quantities of stacked membranes. This suggests, therefore, that the chl-*a/b* LHCII complex is the critical protein enabling the formation of the formation of grana. There is therefore a link between the light harvesting complexes and the thylakoid membrane organisation. In cyanobacteria this link is simple: grana are simply not possible, owing to the phycobilisomes on the membrane being so large.

In higher plants, PSII and PSI are physically separated. It is highly likely that this is of functional importance. Since the capability of PSI to trap excitation energy is far more efficient than that of PSII, it is critical that the two reaction centres are kept apart in order to maintain a flexibility of light harvesting to each, and hence maintain a balance between linear and cyclic electron transport. Further, by stacking membranes where PSII is found, energy can be conserved until a free PSII centre is capable of picking up the harvested light.

In green plants, stacking of the thylakoid membranes is widely observed. Why this may occur is not well understood. It is known that the reaction centres complex, and it may be proposed that PSII aggregation and the interspersal of PSI prevents the loss of energy which has been harvested by the chlorophyll antennae. Psb28 is

conserved in higher plants, and it may be that this is a protein which is involved in maintaining the aggregation, and maximising the ability to conserve energy which has been harvested. The insertional inactivation mutant in *Synechococcus* 7942 (chapter 7) is capable of state transitions (slow ones in the case of state 2), but has massively up-regulated PSII, possibly to compensate for deficiencies in light harvesting, owing to loss of energy from this reaction centre too quickly on account of the randomisation of the rows of dimers.

8.6. Future Implications

The story of how RpaC influences the capability of a cell to carry out state transitions has advanced as a result of work presented in chapters 4 and 5. It is now thought that RpaC is a protein which assists binding of phycobilisomes to PSII (Joshua and Mullineaux, *in press*). It has, however, yet to be confirmed that RpaC is located in the thylakoid membrane of the cell, and the complexes which it purifies with.

Generating a GFP tagged RpaC may help to explain whether, in fact, RpaC is sticking to phycobilisomes in the *Synechocystis* 6803 over-expressor strain, causing its deleterious phenotype (fluorescence quenching under red illumination conditions, slow growth as a result of suboptimal light harvesting of the phycobilisomes to PSII and inability to conduct state transitions). Whether RpaC normally associates with PSII reaction centres is also yet to be elucidated. The GFP-RpaC mutants would also be amenable to FRAP experiments, so information pertaining to the mobility of RpaC could also be obtained. This may suggest whether the binding is happening as a result of illumination triggers or whether it is permanently bound. The use of high osmotic strength buffers could be used to fine-tune how and when binding occurs.

NPQ in cyanobacteria is still not well understood. Data presented here support a hypothesis that phycobilisomes bind to IsiA (or another complex) in iron-stressed cells to trigger a fluorescence decrease upon saturating illumination (Joshua *et al*, 2005). Since it is difficult to confirm that phycobilisomes and their association with certain complexes are specifically required for the quenching phenomenon, similar

experiments on cells possessing mutations of the allophycocyanin core may be carried out. It is known that the APC cores are required for state transitions (Ashby and Mullineaux, 1999a), and that state transitions require phycobilisomes to bind to a specific appropriate degree to reaction centres (Joshua and Mullineaux, 2004; Joshua and Mullineaux, *in press*), it is not unreasonable to suggest that alteration of the APC core may reduce the binding affinity for the phycobilisomes for IsiA. Should this be the case, one might be more convinced that phycobilisomes are indeed binding to IsiA upon induction of NPQ, especially if the recovery was less inhibited in the presence of 1 M phosphate buffers.

It is known that Psb28 copurifies with PSII in plants (Lindahl *et al*, 1995), but it has not yet been established why is only does so occasionally (Ferreira *et al*, 2004), and why there is so much interspecific variability. In order to assist our ability to determine why Psb28 is conserved across phototrophic organisms from cyanobacteria to higher plants, it is imperative that we determine whether inactivation of the gene across a wide range of species gives rise to a similar phenotype. The fact that the gene is conserved suggests its function, too, is conserved. If, as the data presented in this thesis suggests, Psb28 is involved in maintaining the conformation of PSII dimer rows in the thylakoid membrane of cyanobacterial species, it may give the photosynthesis community further information regarding the stacking of higher plant thylakoids into grana, a concept which is yet to be explained satisfactorily.

Freeze fracture electron microscopy of membranes of *Synechocystis* 6803 *psb*28$^-$ and *Synechococcus* 7942 *psb*28$^-$, would confirm or refute the hypothesis that randomisation of the PSII rows occurs in the mutant giving rise to strange state transition phenotypes, slow growth in chlorophyll absorbed blue light and mobile PSII complexes.

Tagging Psb28 with a fluorescently labelled marker, and conducting FRAP experiments, would enable more clear elucidation of whether the protein is permanently fixed in the membrane, associating with PSII dimers (which, in the wild type and growing under non-stressful conditions are known to be immobile (Mullineaux *et al*, 1997; Sarcina *et al*, 2001). Given the suggestion that the plant

version of Psb28 is an extrinsic PSII subunit may explain why it does not always copurify in PSII preparations of cyanobacteria (Ferreira *et al*, 2004), but the work presented in this thesis has not determined whether the cyanobacterial Psb28 acts in the same way, or whether it only associates with PSII as a result of unknown environmental signals. It has also been purported that the protein may be phosphorylated under certain conditions, and this is also yet to be investigated.

Chapter 9: References

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